

JOURNAL OF
EXPERIMENTAL
BOTANY

AN OFFICIAL ORGAN OF THE
SOCIETY FOR
EXPERIMENTAL BIOLOGY

VOLUME 7

OXFORD
AT THE CLARENDON PRESS

1956

Oxford University Press, Amen House, London E.C. 4

GLASGOW NEW YORK TORONTO MELBOURNE WELLINGTON

BOMBAY CALCUTTA MADRAS KARACHI

CAPE TOWN IBADAN NAIROBI ACCRA SINGAPORE

PRINTED IN GREAT BRITAIN

AT THE UNIVERSITY PRESS, OXFORD

BY CHARLES BATEY, PRINTER TO THE UNIVERSITY

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CONTENTS

NUMBER 19

A. ALLSOPP: Morphogenetic Effects of 3-Indolylacetonitrile on Sporelings of <i>Marsilea</i> in Aseptic Culture	1
A. ALLSOPP: Apical Dominance in <i>Marsilea</i> , with Particular Reference to the Effects of 3-Indolylacetic Acid, 3-Indolylacetonitrile, and Coumarin on Lateral Bud Development	14
N. G. BALL and INGA J. DYKE: The Effects of Indole-3-Acetic Acid and 2:4-Dichlorophenoxyacetic Acid on the Growth Rate and Endogenous Rhythm of Intact <i>Avena</i> Coleoptiles	25
S. H. CROWDY, JOHN F. GROVE, H. G. HEMMING, and KATHLEEN C. ROBINSON: The Translocation of Antibiotics in Higher Plants. II. The Movement of Griseofulvin in Broad Bean and Tomato	42
S. E. ARNEY: Studies in Growth and Development in the Genus <i>Fragaria</i> . VI. The Effect of Photoperiod and Temperature on Leaf Size	65
E. M. TURNER: The Nature of the Resistance of Oats to the Take-All Fungus. II. Inhibition of Growth and Respiration of <i>Ophiobolus Graminis</i> Sacc. and other Fungi by a Constituent of Oat Sap	80
A. M. MAYER: The Action of Thiourea as a Germination Stimulator	93
A. G. MORTON: A Study of Nitrate Reduction in Mould Fungi	97
ANNE MACMILLAN: The Entry of Ammonia into Fungal Cells	113
N. SUNDERLAND and R. BROWN: Distribution of Growth in the Apical Region of the Shoot of <i>Lupinus albus</i>	127

NUMBER 20

G. L. HODGSON and G. E. BLACKMAN: An Analysis of the Influence of Plant Density on the Growth of <i>Vicia faba</i> . I. The Influence of Density on the Pattern of Development	147
W. MARY CROMBIE and R. COMBER: Fat Metabolism in Germinating <i>Citrullus vulgaris</i>	166
W. MARY CROMBIE: Fat Metabolism in the West African Oil Palm (<i>Elaeis Guineensis</i>). Part I. Fatty Acid Formation in the Maturing Kernel	181

P. ST. J. EDWARDS and A. ALLSOPP: The Effects of Changes in the Inorganic Nitrogen Supply on the Growth and Development of <i>Marsilea</i> in Aseptic Culture	194
D. D. DAVIES: Soluble Enzymes from Pea Mitochondria	203
S. HOUSLEY and J. A. BENTLEY: Studies in Plant Growth Hormones. IV. Chromatography of Hormones and Hormone Precursors in Cabbage	219
G. BRITTON, S. HOUSLEY, and J. A. BENTLEY: Studies in Plant Growth Hormones. V. Chromatography of Hormones in Excised and Intact Roots of Tomato Seedlings	239
A. D. MCKELVIE: Cherelle Wilt of Cacao. I. Pod Development and its Relation to Wilt	252
H. J. M. BOWEN and J. A. DYMOND: The Uptake of Calcium and Strontium by Plants from Soils and Nutrient Solutions	264
C. P. WHITTINGHAM: Induction Phenomena of Photosynthetic Algae at Low Partial Pressures of Oxygen	273
JOYCE WILKINSON: Some Aspects of Phosphate Nutrition in the Root System of Broad Bean (<i>Vicia faba</i>)	290
E. ROBINSON: Proteolytic Enzymes in Growing Root Cells	296

NUMBER 21

V. H. BLACKMAN: Botanical Retrospect	ix
F. A. L. CLOWES: Localization of Nucleic Acid Synthesis in Root Meristems	307
O. V. S. HEATH and B. ORCHARD: Studies in Stomatal Behaviour. VII. Effects of Anaerobic Conditions Upon Stomatal Movement—A Test of Williams's Hypothesis of Stomatal Mechanism	313
J. CALDWELL: Studies in the Respiration of Apples at Various Pressures of Oxygen	326
S. H. CROWDY and D. RUDD JONES: The Translocation of Sulphonamides in Higher Plants. I. Uptake and Translocation in Broad Beans	335

D. B. IDLE: Studies in Extension Growth. I. A New Contact Auxanometer	347
E. R. TURNER and CHRISTINE E. QUARTLEY: Studies in the Respiratory and Carbohydrate Metabolism of Plant Tissues. VIII. An Inhibition of Respiration in Peas Induced by 'Oxygen Poisoning'	362
B. P. EDDY: The Suitability of Some Algae for Mass Cultivation for Food, with Special Reference to <i>Dunaliella bioculata</i>	372
D. WYNN PARRY: The Effects of 2:4-Dichlorophenoxyacetic Acid, 2:3:5-Tri-Iodobenzoic Acid, and Thiourea on the Vegetative and Reproductive Growth of <i>Avena sativa</i>	381
G. BOND: Some Aspects of Translocation in Root Nodule Plants	387
M. HOLDSWORTH: The Concept of Minimum Leaf Number	395
I. A. M. CRUICKSHANK and T. SWAIN: Study of Phenolic Compounds in Oil-Flax (<i>Linum usitatissimum</i>)	410
I. MANTON and B. CLARKE: Observations with the Electron Microscope on the Internal Structure of the Spermatozoid of Fucus	416

CORRIGENDUM

'The Effects of Indole-3-Acetic Acid and 2:4-Dichlorophenoxyacetic Acid on the Growth Rate and Endogenous Rhythm of Intact *Avena* Coleoptiles'

By N. G. Ball and Inga J. Dyke, Volume 7, No. 19
p. 40, l. 20. For 'does develop.' read 'does not develop.'

INDEX TO VOLUME 7

ALLSOPP, A. Morphogenetic Effects of 3-Indolylacetonitrile on Sporelings of Marsilea in Aseptic Culture	1
ALLSOPP, A. Apical Dominance in Marsilea, with Particular Reference to the Effects of 3-Indolylacetic Acid, 3-Indolylacetonitrile, and Coumarin on Lateral Bud Development	14
ALLSOPP, A., see EDWARDS, P. ST. J.	
ARNEY, S. E. Studies in Growth and Development in the Genus <i>Fragaria</i> . VI. The Effect of Photoperiod and Temperature on Leaf Size	65
BALL, N. G., and DYKE, INGA J. The Effects of Indole-3-Acetic Acid and 2:4-Dichlorophenoxyacetic Acid on the Growth Rate and Endogenous Rhythm of Intact <i>Avena</i> Coleoptiles	25
BENTLEY, J. A., see BRITTON, G.	
BENTLEY, J. A., see HOUSLEY, S.	
BLACKMAN, G. E., see HODGSON, G. L.	
BLACKMAN, V. H. Botanical Retrospect	ix
BOND, G. Some Aspects of Translocation in Root Nodule Plants	387
BOWEN, H. J. M., and DYMOND, J. A. The Uptake of Calcium and Strontium by Plants from Soils and Nutrient Solutions	264
BRITTON, G., HOUSLEY, S., and BENTLEY, J. A. Studies in Plant Growth Hormones. V. Chromatography of Hormones in Excised and Intact Roots of Tomato Seedlings	239
BROWN, R., see SUNDERLAND, N.	
CALDWELL, J. Studies in the Respiration of Apples at Various Pressures of Oxygen	326
CLARKE, B., see MANTON, I.	
CLOWES, F. A. L. Localization of Nucleic Acid Synthesis in Root Meristems	307
COMBER, R., see CROMBIE, W. MARY.	
CROMBIE, W. MARY. Fat Metabolism in the West African Oil Palm (<i>Elaeis Guineensis</i>). Part I. Fatty Acid Formation in the Maturing Kernel.	181
CROMBIE, W. MARY, and COMBER, R. Fat Metabolism in Germinating <i>Citrullus vulgaris</i>	166
CROWDY, S. H., GROVE, J. F., HEMMING, H. G., and ROBINSON, KATHLEEN C. The Translocation of Antibiotics in Higher Plants. II. The Movement of Griseofulvin in Broad Bean and Tomato	42
CROWDY, S. H., and RUDD JONES, D. The Translocation of Sulphonamides in Higher Plants. I. Uptake and Translocation in Broad Beans	335
CRUICKSHANK, I. A. M., and SWAIN, T. Study of Phenolic Compounds in Oil-Flax (<i>Linum usitatissimum</i>).	410
DAVIES, D. D. Soluble Enzymes from Pea Mitochondria	203
DYKE, INGA J., see BALL, N. G.	
DYMOND, J. A., see BOWEN, H. J. M.	
EDDY, B. P. The Suitability of Some Algae for Mass Cultivation for Food, with Special Reference to <i>Dunaliella bioculata</i>	372

EDWARDS, P. ST. J., and ALLSOPP, A. The Effects of Changes in the Inorganic Nitrogen Supply on the Growth and Development of <i>Marsilea</i> in Aseptic Culture	194
GROVE, J. F., see CROWDY, S. H.	
HEATH, O. V. S., and ORCHARD, B. Studies in Stomatal Behaviour. VII. Effects of Anaerobic Conditions Upon Stomatal Movement—A Test of Williams's Hypothesis of Stomatal Mechanism	313
HEMMING, H. G., see CROWDY, S. H.	
HODGSON, G. L., and BLACKMAN, G. E. An Analysis of the Influence of Plant Density on the Growth of <i>Vicia faba</i> . I. The Influence of Density on the Pattern of Development	147
HOLDSWORTH, M. The Concept of Minimum Leaf Number	395
HOUSLEY, S., and BENTLEY, J. A. Studies in Plant Growth Hormones. IV. Chromatography of Hormones and Hormone Precursors in Cabbage	219
HOUSLEY, S., see BRITTON, G.	
IDLE, D. B. Studies in Extension Growth. I. A New Contact Auxanometer	347
MACMILLAN, ANNE. The Entry of Ammonia into Fungal Cells	113
MANTON, I., and CLARKE, B. Observations with the Electron Microscope on the Internal Structure of the Spermatozoid of <i>Fucus</i>	416
MAYER, A. M. The Action of Thiourea as a Germination Stimulator.	93
McKELVIE, A. D. Cherelle Wilt of Cacao. I. Pod Development and its Relation to Wilt	252
MORTON, A. G. A Study of Nitrate Reduction in Mould Fungi	97
ORCHARD, B., see HEATH, O. V. S.	
QUARTLEY, CHRISTINE E., see TURNER, E. R.	
ROBINSON, E. Proteolytic Enzymes in Growing Root Cells	296
ROBINSON, KATHLEEN C., see CROWDY, S. H.	
RUDD JONES, D., see CROWDY, S. H.	
SUNDERLAND, N., and BROWN, R. Distribution of Growth in the Apical Region of the Shoot of <i>Lupinus albus</i>	127
SWAIN, T., see CRUICKSHANK, I. A. M.	
TURNER, E. M. The Nature of the Resistance of Oats to the Take-All Fungus. II. Inhibition of Growth and Respiration of <i>Ophiobolus Graminis</i> Sacc. and other Fungi by a Constituent of Oat Sap	80
TURNER, E. R., and QUARTLEY, CHRISTINE E. Studies in the Respiratory and Carbohydrate Metabolism of Plant Tissues. VIII. An Inhibition of Respiration in Peas Induced by 'Oxygen Poisoning'	362
WHITTINGHAM, C. P. Induction Phenomena of Photosynthetic Algae at Low Partial Pressures of Oxygen	273
WILKINSON, JOYCE. Some Aspects of Phosphate Nutrition in the Root System of Broad Bean (<i>Vicia faba</i>)	290
WYNN PARRY, D. The Effects of 2:4-Dichlorophenoxyacetic Acid, 2:3:5-Tri-Iodobenzoic Acid, and Thiourea on the Vegetative and Reproductive Growth of <i>Avena sativa</i>	381

Morphogenetic Effects of 3-Indolylacetonitrile on Sporelings of *Marsilea* in Aseptic Culture

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Received 20 January 1955

SUMMARY

It is shown that 3-indolylacetonitrile, at 10 mg./l., induces great internode extension in sporelings of *Marsilea*, in aseptic culture, even in media (4 per cent. glucose or 2 per cent. glucose + 2 per cent. mannitol) in which without IAN the internodes are almost completely suppressed. The nitrile also retards the heteroblastic leaf development and inhibits the development of morphological features characteristic of land forms.

The effects of the nitrile on extension growth are discussed in relation to the relevant literature. The other effects of the nitrile are also discussed and it is concluded that they are secondary consequences of the effect of the nitrile on extension growth.

INTRODUCTION

IN previous studies of the effects of cultural conditions on the development of sporelings of *Marsilea* in aseptic culture, it was shown that the sugar concentration has a marked effect on the morphology and anatomy of the sporelings. At relatively high sugar concentrations (e.g. 4 and 5 per cent. glucose) the sporelings even when completely submerged are similar to typical land forms, whereas at relatively low concentrations (1 and 2 per cent. glucose) they have the characteristics of naturally occurring water forms (Allsopp, 1953, 1954b). Further work demonstrated that similar differences could be produced, at a constant nutrient level, by changes in the osmotic concentration of the medium, or by exposure of the shoot to an incompletely saturated atmosphere, and it was concluded that the characteristic features of land and water forms of *Marsilea*, and probably of amphibious plants in general, are determined by the water balance of the developing tissues, nutritional conditions playing only a subsidiary role (Allsopp, 1955). The evidence available, however, although sufficient to establish the fundamental importance of the water balance, provided no clear picture as to the detailed operation of this factor.

A possible approach to this problem was suggested by the observation that the experimentally induced 'land' forms are characterized by a marked absence of internode extension. It seemed not unlikely that some of the features of the 'land' forms might be a secondary response to the suppression of internode elongation. On this hypothesis, it might also be expected that the production of 'water' forms could be achieved by inducing elongation of the internodes of sporelings growing in solutions of an osmotic concentration which would normally lead to the development of 'land' forms. The use of

the naturally occurring growth hormone 3-indolylacetonitrile (IAN) in such an investigation was indicated by the results of a previous study (Allsopp, 1954) in which it was shown that IAN at 10 mg./l. causes great elongation of the internodes in sporelings on the basic 2 per cent. glucose medium. The following account deals with the effects of IAN in media of an osmotic concentration which would normally result in the development of 'land' forms.

MATERIALS AND METHODS

Aseptic cultures of *Marsilea Drummondii* sporelings were prepared from fertilized 'spores' by methods described previously (Allsopp, 1952). The sporelings were grown separately in glass culture tubes containing 10 ml. of the basic inorganic medium with addition of other substances as required by the particular experiment. The temperature in the culture room was regulated as $21^{\circ} \pm 1^{\circ} \text{C}$. and the light intensity (100 f.c. at the level of the cultures) was sufficient to avoid any appearance of etiolation in the sporelings, but was inadequate for growth unless an organic carbon source was provided in the medium.

All media were autoclaved at 110°C . for 20 minutes. Previous work (Allsopp, 1954) had shown that under these conditions there was no detectable hydrolysis of IAN.

Comparison of the effects of different media was always made between sporelings obtained from the same sporocarp. Statistical significance of the numerical results was confirmed by the 't' test after application of Bessel's correction for small numbers.

EXPERIMENTAL RESULTS

I. Effects of IAN in glucose media. The object of this experiment was to make a comparison of the effects of IAN on the development of sporelings in a glucose medium (2 per cent.) which would normally give rise to 'water' forms, and in a glucose medium (4 per cent.) which would normally yield 'land' forms.

Sporelings were grown in media containing in addition to the usual inorganic constituents (a) 2 per cent. glucose, (b) 2 per cent. glucose+1 mg./l. IAN, (c) 2 per cent. glucose+10 mg./l. IAN, (d) 4 per cent. glucose, (e) 4 per cent. glucose+0.1 mg./l. IAN, (f) 4 per cent. glucose+1.0 mg./l. IAN, (g) 4 per cent. glucose+10.0 mg./l. IAN.

The cultures, initially 8 per medium, were grown for several months. Records of the segmentation of the successive leaves, and of the number of leaves per plant are summarized graphically in Figs. 2 and 3. Other numerical data are set out in Table I. Representative sporelings after 3 months of growth are illustrated in Fig. 1.

The effects of IAN in the 2 per cent. glucose media (Figs. 1a, b, c) were exactly as described previously (Allsopp, 1954), i.e. IAN at 10 mg./l. induced great elongation of the internodes and considerable elongation of the petioles. Anatomical examination again confirmed that, as illustrated in the previous paper, the elongation of internode and petiole was due almost entirely to an



FIG. 1. *M. Drummondii*. Sporelings after 3 months of growth on a basic mineral medium with addition of (a) 2% glucose, (b) 2% glucose + 1 mg./l. IAN, (c) 2% glucose + 10 mg./l. IAN, (d) 4% glucose, (e) 4% glucose + 1 mg./l. IAN, (f) 4% glucose + 10 mg./l. IAN. (All nat. size.)

increase in the number of cells in the longitudinal direction. At 1.0 mg./l., IAN induced only slight elongation of internode and petiole.

The effects of IAN on sporelings in the 4 per cent. glucose media were particularly striking. Without IAN the sporelings in 4 per cent. glucose (Fig. 1d) were typical 'land' forms with long slender petioles, relatively large radially disposed leaflets, long thin branching roots, and rhizomes in which the internodes were virtually completely suppressed. With 10 mg./l. IAN (Fig. 1f) there was a remarkable difference in appearance, and the sporelings, without exception, now displayed the characteristics of 'water' forms. The rhizomes had developed long internodes, in fact, as shown in Fig. 1, some of the internodes were as long as the entire rhizomes of sporelings of comparable age in 4 per cent. glucose without IAN. The leaves also had features characteristic of water forms, the petiole being relatively stout and the lamina considerably reduced and lying in the plane of the petiole. The roots were stout and relatively short like those of typical water forms, but had numerous short branches whose growth was soon arrested.

The sporelings from 4 per cent. glucose + 0.1 mg./l. IAN were virtually indistinguishable from those on 4 per cent. glucose without IAN. At 1.0 mg./l. IAN, however, the sporelings (Fig. 1e) although little different from those without IAN showed some trend towards the condition of sporelings in 10 mg./l. IAN; the internodes were still suppressed, but the petioles were rather stouter and the laminae smaller than in the typical land forms from 4 per cent. glucose.

Anatomical examination of certain details of representative sporelings confirmed the conclusions drawn from the external morphology. Attention was first directed to the leaf, in which the distinction between land and water forms is perhaps mostly clearly marked (Allsopp, 1954b, 1955). Leaves of various age from sporelings from the three 2 per cent. glucose media were all typical water forms, i.e. stomata were restricted to the upper epidermis and showed neither overgrowth by the surrounding cells, nor pronounced cuticular thickening. The leaves of sporelings from 4 per cent. glucose without IAN were typical land forms, with numerous sunken, strongly cuticularized stomata on both upper and lower epidermis. In all leaves from sporelings in 4 per cent. glucose + 10 mg./l. IAN, however, the stomata were present on the upper epidermis only and had the other characteristics of water forms. An interesting condition was found in leaves from 4 per cent. glucose + 1 mg./l. IAN; stomata were present on both surfaces, but were fewer on the lower epidermis, evidently as a result of the failure to divide of many of the stomatal mother cells, which could easily be distinguished by their characteristic shape. The complete stomata were again neither sunken nor strongly cuticularized.

The roots of sporelings from all the 2 per cent. glucose media and from the 4 per cent. glucose + 10 mg./l. IAN medium had the tubular prolongations of the cortical cells characteristic of water forms. These tubular processes were lacking in the roots from the 4 per cent. glucose without IAN. In 4 per cent. glucose + 1 mg./l. IAN, most of the roots resembled typical water roots, but

the more recently formed ones had developed the characteristics of land forms.

Examination of the epidermal and cortical cells of the rhizomes of the sporelings from 4 per cent. glucose+10 mg./l. IAN demonstrated that as in the 2 per cent. glucose+10 mg./l. IAN medium, the great elongation of the internodes was due to an increase in cell number in the longitudinal direction rather than to any increase in the length of the individual cells.

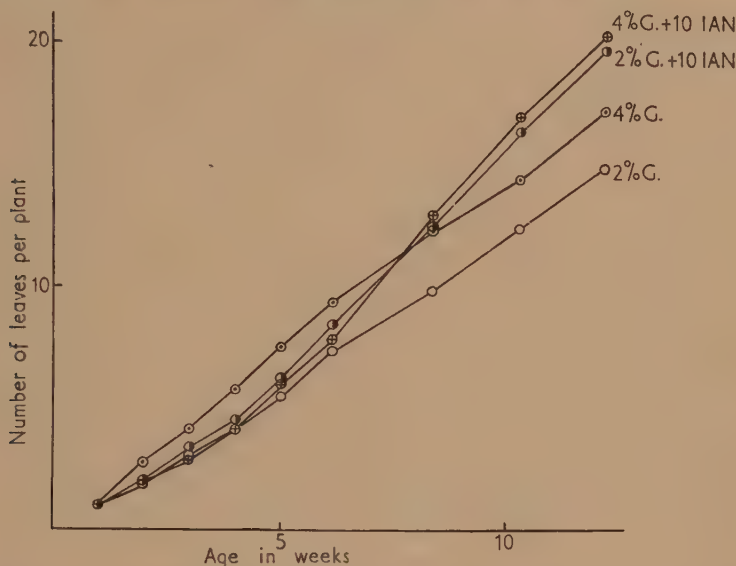


FIG. 2. *M. Drummondii*. Effect of IAN on the rate of leaf formation. Mean values from 7-8 parallel series of cultures. —○— 2% glucose; —●— 2% glucose + 10 mg./l. IAN; —○— 4% glucose; —●— 4% glucose + 10 mg./l. IAN.

Effects on growth and rate of leaf formation. General inspection of the sporelings (Fig. 1) indicates that IAN at 10 mg./l, in addition to producing the morphological changes described, leads to a marked increase in the total amount of growth in the 2 per cent. glucose media, and probably also in the 4 per cent. glucose medium. This impression is borne out by the number of leaves produced per plant (Fig. 2 and Table I). The increase in leaf number in both 2 and 4 per cent. glucose media on addition of 10 mg./l. IAN is statistically highly significant. The increase at 1 mg./l. IAN is not statistically significant but is probably indicative of a real increase.

Effects on leaf segmentation. The effects of IAN on heteroblastic development, as indicated by changes in segmentation of the leaves, are illustrated in Fig. 3. The results are expressed rather differently in Table I, which presents the mean values for the number of the node at which the adult, quadrifid, leaf first appears in each medium.

It is shown that as in previous experiments (Allsopp, 1953, 1955) the heteroblastic leaf development proceeds more rapidly in 4 per cent. than in 2 per

cent. glucose solutions. The differences produced by addition of 0.1 and 1 mg./l. IAN are not statistically significant, but at 10 mg./l. IAN, in both the 2 and 4 per cent. glucose media, there is a considerable and statistically highly significant delay in the completion of heteroblastic development, the adult leaf first appearing at a later node.

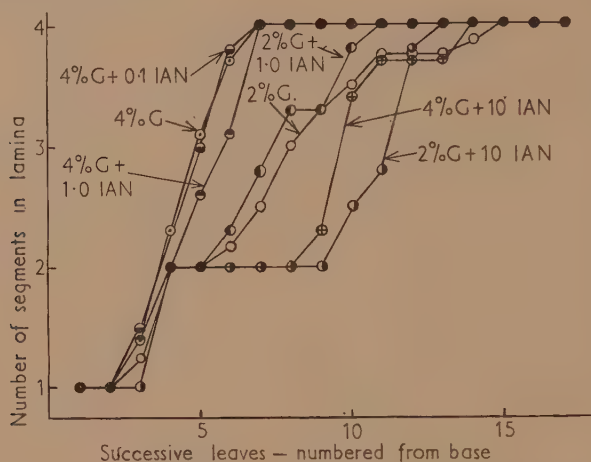


FIG. 3. *M. Drummondii*. Effect of IAN on the segmentation of successive leaves. Mean values from 7-8 parallel series of cultures. —○— 2% glucose; —●— 2% glucose+1 mg./l. IAN; —●— 2% glucose+10 mg./l. IAN; —●— 4% glucose; —●— 4% glucose+0.1 mg./l. IAN; —●— 4% glucose+1.0 mg./l. IAN; —●— 4% glucose+10.0 mg./l. IAN.

TABLE I

Effect of IAN on formation and segmentation of leaves of Marsilea

Medium			Mean values per medium	
Glucose %	IAN mg./l.	No. of sporelings	Node of Insertion of first adult (4-fid) leaf	Number of leaves per plant after 3 months of growth
2	0.0	8	9.13 ± 0.95	15.00 ± 0.70
2	1.0	8	8.50 ± 0.53	16.00 ± 0.49
2	10.0	8	11.50 ± 0.35	19.88 ± 0.45
4	0.0	7	5.43 ± 0.35	17.29 ± 0.74
4	0.1	7	5.14 ± 0.22	17.14 ± 0.42
4	1.0	7	6.14 ± 0.36	17.57 ± 0.48
4	10.0	7	10.57 ± 0.57	20.43 ± 0.57
Glucose %	Mannitol %	IAN mg./l.		
2	0	0	10.86 ± 0.79	16.71 ± 0.72
2	2	0	10.00 ± 1.29	14.40 ± 0.68
4	0	0	6.14 ± 0.69	14.57 ± 0.40
2	2	10	8.83 ± 0.37	16.00 ± 0.41

II. *Effects of IAN in media containing mannitol or glycerol.* The experiments described above demonstrated that IAN at 10 mg./l. prevents the formation

of the 'land' form characteristics which normally appear in a 4 per cent. glucose medium. It seemed of interest to determine whether IAN could also overcome the tendency for the development of 'land' forms in media in which the osmotic concentration had been increased by addition of non-nutrient substances such as mannitol.

Cultures were therefore grown on the following media: (a) 2 per cent. glucose, (b) 4 per cent. glucose, (c) 2 per cent. glucose+2 per cent. mannitol, (d) 2 per cent. glucose+2 per cent. mannitol+10 mg./l. IAN, (e) 1 per cent. glycerol, (f) 2 per cent. glucose+1 per cent. glycerol, (g) 2 per cent. glucose+1 per cent. glycerol+10 mg./l. IAN.

Originally ten 'spores' were transferred to each medium, but their viability was rather low and no more than 7 embryos developed in any medium. Some of the results are presented in the lower half of Table I, and are illustrated in Fig. 4.

As usual, the sporelings from the 2 per cent. glucose medium were all 'water' forms, while of the 7 sporelings from the 4 per cent. glucose, 6 were typical 'land' forms (Fig. 4a). In the 2 per cent. glucose+2 per cent. mannitol medium, only 5 sporelings survived, but confirmed the previous results (Allsopp, 1955) that on this medium the sporelings are either small but otherwise typical 'land' forms or stunted abnormal-looking plants with short internodes; in the present experiment 3 were 'land' forms (Fig. 4b) and 2 stunted sporelings. The results in the 2 per cent. glucose+2 per cent. mannitol+10 mg./l. IAN medium could perhaps have been predicted from the effects of IAN in the 4 per cent. glucose medium. In the mannitol medium also, addition of 10 mg./l. IAN resulted in great elongation of the internodes and loss of all 'land' form characteristics (Fig. 4c). The values for the insertion of the adult leaf recorded in Table I, confirm the finding (Allsopp, 1955) that although addition of 2 per cent. mannitol to a 2 per cent. glucose medium results in the development of sporelings with 'land' form characteristics yet there is no increase in the rate of heteroblastic development. The sporelings are stunted and as shown in Table I the rate of leaf formation falls below that of the 2 per cent. glucose medium. Addition of IAN, besides increasing the total amount of growth, raises the rate of leaf formation to that of the 2 per cent. glucose medium without mannitol.

Glycerol alone was not a suitable carbon source, and although seven sporelings commenced growth they produced only simple spatulate leaves and eventually died. In the presence of 2 per cent. glucose more growth was made, but the plants were still stunted and distorted with no internode development. With 2 per cent. glucose+1 per cent. glycerol+10 mg./l. IAN better growth was obtained, but the sporelings were of irregular abnormal appearance although there was appreciable development of internodes.

III. *Effects of IAN in transfer experiments.* In the experiments described in the two preceding sections, sporelings were exposed to the action of IAN from the earliest stages of embryo development. In the present section IAN was supplied to sporelings which had already undergone development for three

months in a 4 per cent. glucose medium without IAN and had acquired 'land' form characteristics.



FIG. 4. *M. Drummondii*. *a, b, c*, sporelings after 5 months on a basic mineral medium with addition of (*a*) 4% glucose, (*b*) 2% glucose+2% mannitol, (*c*) 2% glucose+2% mannitol + 10 mg./l. IAN. (All nat. size.)

d, e, f. Sporelings 1 month after transfer, following growth for 3 months on a 4% glucose medium to (*d*) 4% glucose+10 mg./l. IAN, (*e*) 2% glucose+2% mannitol, (*f*) 2% glucose+2% mannitol+10 mg./l. IAN. (All $\frac{2}{3}$ nat. size.)

The sporelings were transferred to three different media (6 sporelings per medium) consisting of the basic inorganic constituents with additions of (*a*) 4 per cent. glucose+10 mg./l. IAN, (*b*) 2 per cent. glucose+2 per cent. mannitol, (*c*) 2 per cent. glucose+2 per cent. mannitol+10 mg./l. IAN. The subsequent development of the sporelings was different and characteristic in

the three media. Representative sporelings, a month after transfer, are illustrated in Figs. 4*d*, *e*, and *f*.

The sporelings transferred to 2 per cent. glucose+2 per cent. mannitol confirmed previous experiments of this type (Allsopp, 1955), i.e. there is no immediate change in morphological characteristics when sporelings are transferred from a medium of high sugar concentration to one of low sugar concentration, but with the total osmotic concentration maintained at the original level by addition of mannitol. In the two media with IAN, however, the parts formed after transfer differed from those already present. In both media there was considerable elongation of the rhizome internodes, although this was more marked in the 4 per cent. glucose than in the glucose+mannitol medium. The growth rate, as indicated both by rhizome length and by number of leaves produced was also greater in the 4 per cent. glucose medium than in either of the mannitol media (the mean values for the number of leaves produced during the month following transfer were 3.8 ± 0.44 in the 2 per cent. glucose+2 per cent. mannitol medium; 5.0 ± 0.35 in the 2 per cent. glucose+2 per cent. mannitol+10 mg./l. IAN, and 6.4 ± 0.46 in the 4 per cent. glucose+10 mg./l. IAN).

The illustrations in Fig. 4 might suggest that the leaves formed after transfer were typical land forms. Closer examination revealed that this was so only in the 2 per cent. glucose+2 per cent. mannitol medium, in which the leaflets had numerous fully differentiated stomata on both surfaces. In the two media containing IAN, the leaves had the delicate flexible petioles characteristic of the floating leaves and certain types of submerged leaves of *Marsilea*. In the lamina, the stomata were numerous in the upper epidermis and almost or entirely absent from the lower. The experiment was interrupted at a time when transfer to larger containers would have become necessary, but it seems not unlikely that in the IAN media reversion to even less differentiated types of leaf would have eventually been achieved. As in the experiments of sections I and II the considerable increase in internode length was determined by an increase in cell number; not by increase in the length of the individual cells.

DISCUSSION

From the above account it may be concluded that IAN has three principal effects on the development of sporelings of *Marsilea* in aseptic culture: (*a*) on longitudinal growth, particularly of the internodes, (*b*) on heteroblastic development, and (*c*) on the appearance of land or water form characteristics.

(*a*) *Effect of IAN on longitudinal growth.* The great increase in internode extension produced by IAN at a suitable concentration (10 mg./l.) is apparently in agreement with the known effects of IAN (Bentley and Housley, 1952) and other auxins in promoting the longitudinal growth of oat coleoptiles and other plant organs. But the increased growth observed in the majority of studies of auxin action is a result of cellular elongation, whereas in the present work with *Marsilea* there was no appreciable increase in cell length, and the great extension of the internodes was due to an increase in the number of cells in the

longitudinal direction. It is possible that the effect of IAN on *Marsilea* may be more representative of the normal action of auxins in intact plants, for the materials most frequently used in auxin studies are either oat coleoptiles, which are organs with rather special properties and restricted systematic distribution, or other relatively mature tissues in which cell division has already ceased. The mechanism of elongation, however, may be fundamentally the same in the two cases, with the increase in cell number a secondary consequence of increased elongation of actively dividing cells.

It is widely held (Bonner and Bandurski, 1952) that the outstanding feature of auxin-induced growth is an increased uptake of water; but there is less agreement as to the mechanism of the process. Largely as a result of studies on the effects of auxin on the uptake of water by storage tissues, such as potato tuber, a number of workers (e.g. Commoner, *et al.* 1943; Bonner and Bandurski, 1952; Bonner, *et al.*, 1953; Bogen, 1954; Hanson and Bonner, 1954) have concluded that there is an active water uptake controlled by respiratory or related activities of the cell. Other workers have convincingly shown, however, that a change in the extensibility of the cell walls, probably also under protoplasmic control, is sufficient to account for the effects of auxin on water uptake (Brauner and Hasman, 1952; Burström, 1953, 1953a) while Levitt (1953) presents theoretical arguments against active water absorption. The work of Burström (1953a) with Jerusalem artichoke is particularly valuable in explaining the absorption of water from supposedly hypertonic solutions (Commoner, *et al.*, 1943; Bonner, *et al.*, 1953). He showed that apart from any auxin effects the tubers possessed a rather accurate mechanism of osmoregulation, whereby there was an increase in internal osmotic concentration with any increase in the concentration of the external solution. Such an osmoregulation coupled with an increase in cell wall extensibility might account in large part for the observation in the present work that IAN at 10 mg./l. can induce internode extension in media of an osmotic concentration (4 per cent. glucose and 2 per cent. glucose + 2 per cent. mannitol) in which the internodes are normally suppressed.

The effect of auxin in producing changes in the permeability of the protoplast is also of importance in any consideration of auxin induced growth. Pohl (1948) as a result of studies on the plasmolysis and deplasmolysis of coleoptiles, concluded that the promotion of growth by auxin is due to an increase in water permeability. Guttenberg and Beythien (1951) arrived at a similar conclusion from their studies on *Rhoeo* epidermis cells. Bogen (1954) is correct in pointing out that such changes in water permeability, under otherwise constant conditions, would not influence the final equilibrium, but only the rate of its attainment. This objection would appear to be true for relatively mature tissues, but in rapidly growing tissues the rate of elongation might be limited by the rate of water entry, which could therefore affect the ultimate length of the cell or organ if the cell membrane became resistant to further expansion before the maximum cell size had been attained.

Another important effect of auxin is the increase in permeability to sugars,

demonstrated by Meinel and Guttenberg (1952). Although it is known that cellular elongation is possible without extracellular supplies of nutrients, yet under normal conditions, the elongation of an organ is accompanied by a considerable increase in the amount of protein and other cellular constituents (Burström, 1951). An increase of protoplasmic permeability in response to physiological concentrations of auxin would facilitate the entry of the nutrients required for such a synthesis.

From a survey of the literature in conjunction with the present experimental results, it seems not unlikely that an increase in cell-wall extensibility, coupled with an increase in protoplasmic permeability, not only to water but also to various nutrients, is responsible, for the increased longitudinal growth of *Marsilea* rhizomes, and probably of similar actively growing organs, in response to appropriate concentrations of IAN. Under the conditions of the present experiments, the synthetic activities of the cells and their accompanying division keep pace with cellular elongation, leading to a great increase in cell number, but no appreciable increase in cell length.

Apart from the effect of IAN in promoting extension growth in certain organs, there is an undoubted increase in the total amount of growth as indicated not only by the greater size of the plants but also by an increase in the number of leaves per plant in the presence of IAN. The greater permeability of the protoplasm to sugar in the presence of auxin (Meinel and Guttenberg, 1952) provides an adequate explanation of the improved growth. This effect, although pronounced in culture, is probably of less importance under natural conditions. Changes in protoplasmic permeability under the influence of auxin may be of considerable importance, however, in controlling the distribution of nutrients *within* the plant, as suggested already in a discussion of apical dominance (Allsopp, 1956).

(b) *Effect of IAN on heteroblastic development.* From previous work on *Marsilea*, it was concluded (Allsopp, 1954a) that the heteroblastic development is largely controlled by the nutritional status of the shoot apex. In the present work the heteroblastic development of the leaves was retarded in media containing IAN in contrast with the increase in the rate of leaf formation and in the amount of rhizome growth. This result is not in conflict with the above hypothesis, for the greater growth in IAN was not in the radial dimensions of the rhizome, but in the longitudinal direction only, and rapid transverse divisions in the subapical region might actually impair the nutrition of the more terminal region where the leaf primordia are first formed. Baldovinos (1953) arrived at a similar conclusion from his studies on the growth of maize roots, where the most active cell division is in a region of rapid cell enlargement behind the terminal mm. of the apex.

(c) *Effect of IAN on development of 'land' or 'water' form characteristics.* Morphologically one of the more interesting results of the present investigation was the failure of certain 'land' form characteristics to develop on addition of IAN to media in which such characteristics would otherwise have appeared. This result suggests that the usual appearance of the features of land forms in

response to a water deficit (increased diffusion pressure deficit) of the external medium (Allsopp, 1955) is not a response to a change in the internal osmotic concentration as such, but rather to an associated change in concentration of some nutrient substance, probably sugar. On this view, the great increase in the longitudinal growth of the rhizome, in media containing IAN, would tend to use up and dilute the supply of nutrients so that only a reduced concentration would be available for the additional cellular differentiation characteristic of land forms. In naturally occurring water forms, the greater extension growth accompanied by a reduced level of differentiation would result largely from the absence of any appreciable diffusion pressure deficit in the external medium and perhaps partly from the effects of increased hydration of the protoplasmic or membrane constituents. The increased development of the lateral organs when the growth of the main stem is suppressed, as in the 'land' forms of *Marsilea*, is probably an example of 'compensatory growth' similar to that described by Jacobs and Bullwinkel (1953) for *Coleus* plants.

From the above discussion of the effects of IAN it may be concluded that the increase in longitudinal growth is the primary one, and that the changes in heteroblastic development and the loss of 'land' form characteristics are secondary consequences of this growth. The view that increased longitudinal growth is responsible for certain characteristics of water forms, provides an extension of a similar view adopted by Loomis (1953, p. 197) to account for reduced differentiation in rapidly growing land plants.

ACKNOWLEDGEMENTS

I am indebted to Professor C. W. Wardlaw for his interest in the work and for providing the necessary facilities. I have also to thank Mr. E. Ashby for the photographs in Figs. 1 and 4, and Dr. J. A. Bentley for the sample of IAN.

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Apical Dominance in *Marsilea*, with Particular Reference to the Effects of 3-Indolylacetic Acid, 3-Indolylacetoneitrile, and Coumarin on Lateral Bud Development

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Received 7 December 1954

SUMMARY

An account is given of the effects of 3-indolylacetic acid, 3-indolylacetoneitrile, and coumarin on the development of the lateral buds of intact plants, decapitated plants, and excised nodes of *Marsilea Drummondii* in aseptie culture.

In all the plant materials used, lateral buds grew out at all physiological concentrations of the two auxins, an observation which is considered to refute the hypothesis that correlative inhibition is caused by direct inhibition by auxin. Other hypotheses and recent work on the subject are also discussed.

INTRODUCTION

THE problem of apical dominance and the correlative inhibition of lateral buds has received considerable attention, but there is still no agreement as to the processes involved. The original discovery of Thimann and Skoog (1933, 1934) that the action of the terminal bud can be replaced by application of auxin to the cut surface of a decapitated shoot has been abundantly confirmed, although many explanations have been advanced to account for this result. In his comprehensive review Thimann (1939) discusses nine hypotheses, which may be reduced to three distinct points of view:

- (a) the auxin acts directly on the lateral buds (Thimann, 1937);
- (b) the auxin brings about some process which gives rise to a special inhibiting influence (Snow, 1937);
- (c) the auxin leads to a diversion of nutrients or growth factors (Went, 1936).

Of these three principal hypotheses of correlative inhibition, Thimann's view of direct inhibition is probably the most widely accepted. On this view 'the influence of auxin on different organs is represented by a series of optimum curves, intermediate concentrations promoting growth and higher concentrations inhibiting it. The concentrations causing stem growth would be high enough to inhibit bud development' (Thimann, 1952, p. 36.) It might therefore be expected that application of an appropriate range of auxin concentrations direct to the lateral buds would provide a ready means of testing

this hypothesis. Such experiments have been carried out by a number of workers with widely varying interpretation of the results (see Discussion). Since this lack of agreement is probably to be referred to the difficulties inherent in the methods employed rather than to any real difference in the nature of the response, a renewal of the approach with more suitable plant materials seemed desirable. In the present work, the use of *Marsilea* sporelings in aseptic culture eliminated some of the difficulties usually encountered. It is considered that the results obtained clearly refute the hypothesis of direct inhibition. Other hypotheses will be considered in the Discussion.

EXPERIMENTAL METHODS AND RESULTS

The plant materials used were all obtained from aseptic cultures of *Marsilea Drummondii* A.Br., which were grown under the conditions described in previous papers (Allsopp, 1952, 1954). In this earlier work it was shown that these plants are very suitable for the study of the effects of a variety of growth substances.

Like other plants which grow well in liquid media, the submerged cultures of *Marsilea* have the great advantage that it is easy to supply nutrients or growth factors in the external medium without any other change in the conditions of growth. Furthermore, since aquatic plants absorb readily over their whole surface, there is no restriction in the access of the added substances to the cells. Growth under aseptic conditions has the additional advantage that in experiments with excised organs such as buds, external supplies of organic nutrients enable growth to continue even in the absence of considerable masses of storage tissue.

In studies of the effects of auxins on bud development in land plants, on the contrary, it is rarely possible to form a clear picture as to what concentration of the auxin is reaching the living cells. The auxin is often applied in a lanolin paste at a high concentration. Cells in contact with the paste are thus exposed to high concentrations while the internal cells may receive very low concentrations indeed. Apart from the question of auxin concentration, application of the paste itself must result in changed conditions for the underlying cells. Application of solutions of auxin must also change the cellular environment. It is realized that valuable results have certainly been obtained by the use of these methods, but the difficulties mentioned are probably responsible for the varied results of different workers.

Bud development on decapitated plants and excised nodes. The apical dominance of *Marsilea* in liquid culture is quite comparable with that observed in most rhizomatous land plants. As described previously (Allsopp, 1953) the lateral buds, one at the base of each leaf, normally remain dormant unless the main rhizome apex is damaged or removed. In such circumstances activity begins in nearly all the laterals, but the one (or occasionally two) nearest the apex quickly gains the ascendancy and the others are then suppressed. When the rhizome is cut into segments, however, each containing one node with its

dormant lateral bud ('excised nodes'), a new plant is usually produced from the lateral bud of each segment.

The effect of the subtending leaf and of the associated roots at each node has now been studied in three parallel series of cultures of excised nodes: (a) with leaf and roots; (b) with only the leaf removed; and (c) with leaf and roots removed. The nodes were obtained from three aseptic *Marsilea* sporlings which had been grown on the standard basic medium (mineral nutrients + 2 per cent. glucose) for several months and had produced 21–23 leaves. Only the first 10 or 11 nodes, from the apex, were used and as nearly as possible each type of node included equal numbers of similar age, from the three plants. The excision of nodes and organs was carried out in sterile petri dishes and isolated nodes were each transferred to culture tubes containing 10 ml. of the basic medium. Regular observations were made of the development of the lateral buds.

Some of the experimental data are set out in the first column of Table I. It is shown that the lateral bud grew out from every node which survived the operation of excision. In certain plants previous workers have found (cf. Thimann, 1952, p. 38) that the lateral bud is inhibited by its subtending leaf, but under the particular experimental conditions used here, there was no evidence of any such inhibition in *Marsilea*. Similarly, there was no evidence of any inhibition by the roots, although Libbert (1954a) has recently claimed that the roots of *Pisum sativum* play an important role in the inhibition of lateral buds. In *Marsilea*, examination of the nodes 4 days after excision revealed that considerable development had taken place in all the lateral buds, but that those on the nodes without leaves or roots lagged appreciably behind the others. This initial lag was probably merely nutritional, and was soon overcome when the lateral had developed its own roots and leaves thus augmenting its absorbing (and perhaps synthesizing) area. After 6 weeks there was no detectable difference between the laterals from the three treatments.

The results also confirmed the previous conclusion (Allsopp, 1953) that the segmentation of the first leaf produced by a lateral bud is already determined by the position of the bud on the rhizome, although that of the subsequent leaves may be determined by nutritional or other experimental conditions to which the lateral is exposed. In the present experiment, the only laterals in which the segmentation of the first leaf was lower than the quadrifid condition were borne by the eleventh node from the apex. Reversion to bifid or even spatulate leaves, followed by recovery to the quadrifid condition, was fairly frequent in the laterals from the nodes without leaves or roots, probably as a consequence of a temporary nutritional inadequacy. The example of reversion illustrated in Fig. 1a developed from an injured bud on a node bearing both leaf and roots.

Effects of 3-indolylacetic acid, 3-indolylacetoneitrile, and coumarin. The three substances used in the present study have all played an important role in the investigation of plant-growth responses. Indolylacetic acid (IAA) is generally accepted as an important plant-growth hormone (Bonner, 1950, Thimann,

1952); indolylacetonitrile (IAN) is a naturally occurring hormone recently isolated from cabbage (Jones, *et al.*, 1952) and detected since in a considerable

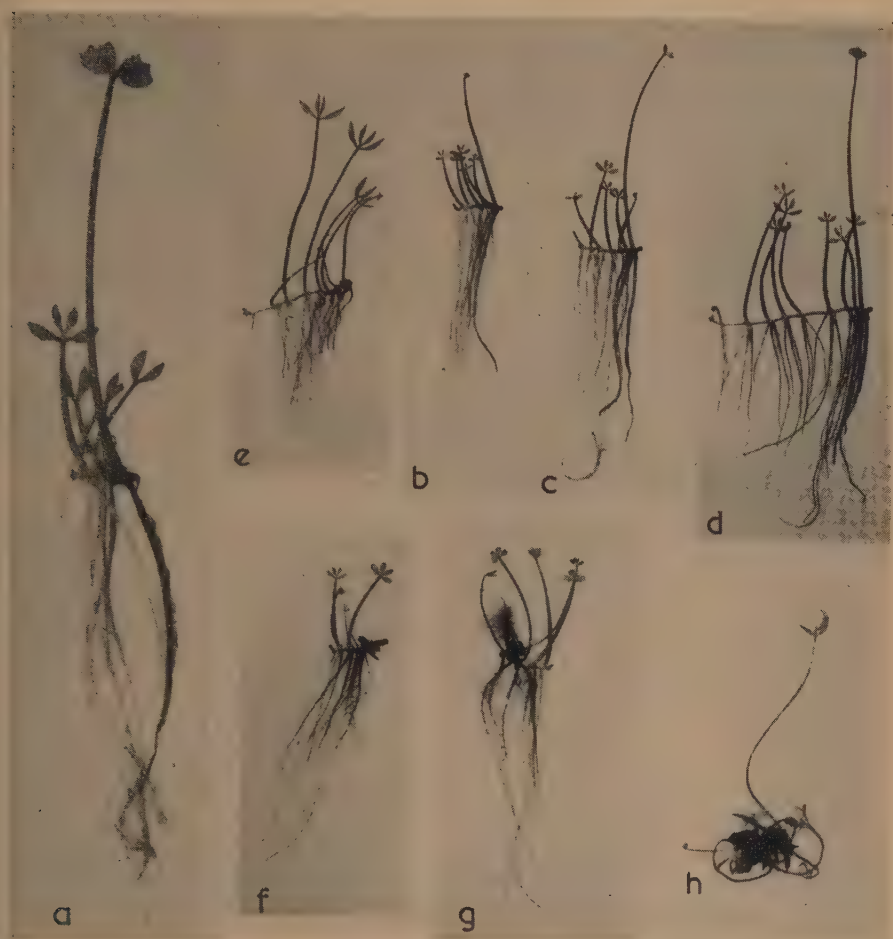


FIG. 1. *M. Drummondii*. Growth of buds 5 weeks after excision. (a) Reversion to juvenile leaves in lateral from injured bud on basic (2% glucose) medium. (b) (c) (d). Laterals from excised nodes, complete with leaf and roots, on basic medium + (b) 0.1, (c) 1.0, and (d) 10 mg./l. IAN. (e) Growth from excised rhizome apex on 4% glucose medium + 10 mg./l. IAN. (f) (g) (h). Laterals from excised nodes, minus leaf and roots, on basic medium + (f) 0.1, (g) 1.0, and (h) 10 mg./l. IAA. Figs. (a) and (h) $\times 2$. Other Figs. Natural size

range of plant materials (Bennet-Clark and Kefford, 1953), while coumarin is well-known as an inhibitor of plant growth and has already been used in an extensive research on correlative inhibition (Libbert, 1954a). An examination has been made in the present work of the effects of these substances on (a) intact plants, (b) decapitated plants, and (c) excised nodes of *Marsilea*.

(a) *Effects on intact plants*. The effects of IAA and IAN on *Marsilea* have

been described in earlier papers (Allsopp, 1952, 1954). For the present purpose it is only necessary to recall that over the whole range of physiological concentrations of these hormones, there was no change in the correlative inhibition of lateral buds.

In the present research, observations were made on the effects of coumarin on the early and subsequent stages of sporeling development. One-day embryos, ten per medium, were transferred to: (a) the basic 2 per cent. glucose medium; (b) the basic medium+10 mg./l. coumarin; (c) the basic medium+100 mg./l. coumarin; and (d) the basic medium+1,000 mg./l. coumarin. At the highest coumarin concentration there was no trace of embryo development; at 100 mg./l. coumarin there was slight development, but the embryos died without further growth, while in 10 mg./l. coumarin development was at least as vigorous as in the glucose controls. The sporelings were similar to the controls in morphological features except that the roots were strikingly better developed in the coumarin medium; they were markedly thicker, more branched, and with more conspicuous root hairs than in the basic glucose medium. This effect of coumarin has apparently not been reported previously, and it is hoped to study it in further detail at a later date. Apical dominance was unaffected in the coumarin medium.

(b) *Effects on decapitated plants.* The plants used in this set of experiments had been grown in culture for 15 weeks and bore approximately twenty leaves. Immediately after excision of the apex, the plants were transferred to culture tubes containing the basic 2 per cent. glucose medium with additions of (a) 1.0 mg./l. IAA, (b) 10 mg./l. IAA (c) 10 mg./l. IAN, (d) 10 mg./l. coumarin, (e) 100 mg./l. coumarin, and (f) 1,000 mg./l. coumarin.

All plants survived the transfer with the exception of those on the two higher coumarin concentrations. In the highest coumarin concentration there was no trace of lateral development. In the 100 mg./l. coumarin concentration, however, there was slight development of the laterals near the apex, but this growth was soon checked, and the plants eventually perished. The results with the medium containing 10 mg./l. coumarin were essentially similar to those obtained with decapitated plants on a 2 per cent. glucose basic medium. Of the six plants used, all produced lateral shoots from the buds nearest the cut surface: two plants had each only one lateral, two had two laterals, and two had two laterals with one clearly dominant. As in the cultures described in section (a) the roots of the cultures on the coumarin medium were more strongly developed than those in the basic medium.

The decapitated plants in the two concentrations of IAA were also similar in behaviour to decapitated plants on the basic media in that all produced lateral shoots from the buds nearest the apical cut surface. In 1 mg./l. IAA each of the six plants produced only one lateral. In 10 mg./l. IAA one plant had one, four had two, and one had three laterals. The response of the roots to IAA was similar to that reported previously for developing sporelings (Allsopp, 1952, 1954). Thus, at 1 mg./l. IAA the roots were of normal length but with numerous laterals, while at 10 mg./l. IAA the roots were stunted and

thickened but still had numerous laterals. The higher IAA concentration was certainly less toxic than in the case of the young sporelings, possibly on account of a greater capacity for IAA inactivation in the larger older plants.

Similar results were obtained with IAN at 10 mg./l. Of the six plants, three produced one lateral, and the other three, two laterals. The laterals all showed the great internode extension characteristic of growth on IAN at 10 mg./l.

Effects on excised nodes. The nodes used in the present experiment were obtained from 5 aseptic sporelings which had been growing for several months and had produced between 21 and 23 leaves. Only the first 12 nodes were used. After excision the nodes were transferred to the 2 per cent. glucose medium with additions of 0.1, 1.0, and 10 mg./l. of IAN or IAA. Each medium received 10 nodes, of which three were complete with leaf and roots, four had only roots, and three had neither leaf nor roots. The treatments are outlined in Table I, which also includes some of the principal results.

TABLE I

Effects of various concentrations of 3-indolylacetic acid and 3-indolylacetonitrile on the development of lateral buds of Marsilea

Medium	Basic medium (2% glucose) only	Basic medium + 0.1 mg./l. IAN	Basic medium + 1 mg./l. IAN	Basic medium + 10 mg./l. IAN	Basic medium + 0.1 mg./l. IAA	Basic medium + 1 mg./l. IAA	Basic medium + 10 mg./l. IAA
Number of nodes with leaf and roots	11	3(1)	3	3	3	3	3(2)
Number of nodes with roots only	10(1)	4	4	4(1)	4(1)	4(1)	4(1)
Number of nodes without leaf or roots	11(3)	3	3	3	3(1)	3	3(1)
Total number of nodes per medium	32	10	10	10	10	10	10
Number of surviving nodes	28	9	10	9	8	9	6
Number of nodes with outgrowth of bud	28	9	10	9	8	9	6

(Numbers in brackets represent number of nodes per batch which died under treatment.)

The results were decisive. In every surviving node, the lateral bud developed into a lateral branch and the morphological responses were such that there was no question of the added growth substances not having gained access to the active cells of the bud. Representative results from the IAN series are illustrated in Figs. 1 *b*, *c*, and *d*. The lateral from the 10 mg./l. IAN medium shows the striking elongation of the internodes previously reported for sporelings in this concentration of IAN (Allsopp, 1954). A similar elongation of the internodes was found in excised apices grown at the same IAN concentration (Fig. 1 *e*).

The lateral buds developed in all concentrations of IAA (Fig. 1 *f*, *g*, *h*) and showed the morphological responses already described for developing sporelings (Allsopp, 1952, 1954). At the lowest concentration, 0.1 mg./l., development of the lateral was similar to that normally found in the basic 2 per cent. glucose medium, apart from the formation of numerous branches on the first few roots of the lateral branch. At 1.0 mg./l. the roots were more markedly

branched and very hairy, while at 10 mg./l. toxic effects were evident, the irregularly swollen laterals bearing malformed leaves and stunted much-branched roots. It is emphasized that in spite of this pathological appearance the lateral buds had undergone extensive development, and in fact eventually recovered and produced normal branches. The four buds which failed to survive the treatment were on the older nodes of the batch, and were evidently more sensitive to the toxic action of the IAA.

Excised nodes in media containing 100 mg./l. and 1,000 mg./l. of coumarin perished like the sporelings and decapitated plants. Excised *apices* in the basic medium with 10 mg./l. coumarin underwent normal development, apart from the particularly vigorous root development already described for sporelings and decapitated plants in this concentration of coumarin. In media containing 1,000 mg./l. coumarin, the apices perished immediately, while with 100 mg./l. there was slight abnormal growth comparable with that from the laterals of excised nodes and decapitated plants.

DISCUSSION

The hypothesis of direct inhibition requires that concentrations of auxin which promote the elongation of the main shoot, should also prevent the outgrowth of lateral buds. Accordingly, since both IAA and IAN exert their maximum effect on cell elongation over the concentration range 0.1 to 10 mg./l. (Bentley and Housley, 1952) it would be expected, as indicated by Thimann (1937, 1952, Fig. 7), that growth of the lateral buds would be completely suppressed over this range of concentrations of the two auxins. It has indeed been claimed that the hypothesis of direct inhibition is supported by the results of Plch (1936) and Thimann (1937) who obtained reduced growth of lateral buds of *Pisum sativum* by direct application of auxin-lanolin paste, and by the results of Skoog (1939) who obtained a similar inhibition in buds of *Pisum* growing in White's root-culture medium. It seems significant, however, that in these experiments the buds *did grow*, even at high auxin concentrations, whereas in the correlative inhibition of intact plants there is *complete arrest* of bud development at an appreciably lower internal auxin concentration. This criticism is supported by the results of other workers. Hemberg (1949) found no inhibition of sprouting when pieces of potato tuber carrying an individual bud were grown in solutions containing from 0.01 to 1.0 mg./l. IAA. Recently, Libbert (1954, 1954a) obtained growth of lateral buds when isolated nodes of *Pisum sativum* were placed in solutions containing 1 mg./l. IAA. By careful application of auxin paste to the youngest leaves of the bud, he was also able to achieve renewal of growth in a main apex which had been inhibited by the stronger growth of an experimentally induced lateral. Snow and Snow (1937) had previously found that application of auxin paste to exposed stem apices of *Lupinus albus* and *Epilobium hirsutum* increased the development of leaf primordia and associated axillary buds.

The evidence from experiments involving the direct application of auxin to individual buds is thus opposed to the hypothesis of direct inhibition. As

detailed in the experimental part of the present paper, the work with *Marsilea* eliminates the possible objection to some of the investigations outlined above, that there is no indication of the concentration of auxin available to the active cells of the bud. With IAN in particular, there was no inhibition of lateral bud growth at the concentration (10 mg./l.) which produced great elongation of the internodes of both lateral and principal rhizomes. This observation lends no support to the suggestion of Gordon (1954) that the failure to obtain inhibition of axillary buds when IAN was applied to decapitated plants of *Phaseolus multiflorus* (Bentley and Bickle, 1952) was due to inability of the stem of this species to hydrolize the nitrile to IAA.

Other well-established facts are in conflict with the hypothesis of direct inhibition. It has been shown repeatedly that the auxin content of inhibited buds or shoots is lower than that of actively growing parts (e.g. Le Fanu, 1936; Ferman, 1938; Van Overbeek, 1938; Libbert, 1954). Loomis (1953) also points out that shoots growing in darkness may be very low in auxin, but inhibition on etiolated shoots is typically complete. Again, shoots making a rapid growth because of favourable growing conditions tend to be high in auxin, but inhibition is relatively low.

Of the remaining hypotheses of correlative inhibition, the views of Snow (1937, 1940) have recently received considerable support from work on plant-inhibiting substances. Snow (1937) elaborated the hypothesis that the auxin moving down the stem leads to the release of a secondary inhibiting influence which arrests the growth of the lateral buds. In his later work (1940) he considered that the secondary influence is probably another plant hormone and that the fate of any part of the shoot system depends on two variables, namely the relative amounts of auxin and inhibitor which it receives, and its inherent sensitivity. Recent work has in fact shown that growth inhibitors are widely distributed in plants (Bennet-Clark and Kefford, 1953). It has also been shown that such inhibitors play a role in the similar phenomenon of dormancy in both potato tubers and winter buds of *Fraxinus* twigs (Hemberg, 1949, 1949a, 1952; Blommaert, 1954). The inhibitors disappear at the end of the rest-period when sprouting of the buds takes place.

More direct support for the views of Snow is provided by the extensive work of Libbert (1954, 1954a) on correlative inhibition in *Pisum*. Libbert advanced the hypothesis that the root system supplies an unsaturated lactone to the shoot, where auxin by some as yet undecided process converts the lactone to a specific inhibitor. Some of the results of Libbert, to which brief reference has already been made, are clearly opposed to the hypothesis of direct inhibition by auxin, but his views on the importance of the root seem to rest largely on the greater inhibition obtained when rooted plants, as compared with unrooted cuttings, are placed in contact with solutions of IAA. This result, however, is perhaps explained merely by a greater absorption of IAA in the rooted plants, for in the present work on *Marsilea*, where absorption of nutrient, &c., can take place over the whole surface of the plant, there was no increase in the inhibition of the lateral buds on the nodes provided with roots.

The lack of inhibition by the roots is not at variance, however, with the general position adopted by Snow.

Although neither the existing literature nor the present work provides any strong arguments against Snow's hypothesis, there is also no evidence available which will disprove the 'diversion' hypothesis of Went (1936, 1939). This view is similar to that of the older morphologists (e.g. Goebel, 1900) who considered that the main apex is an attraction centre, inhibiting the lateral buds by diverting from them their necessary nutrients. Modern views of this type consider that the diversion is brought about by the action of auxin. Support for this view is provided by the results of a number of workers who have shown (as summarized by Wardlaw, 1952, p. 254) that IAA promotes translocation of various nutrients to the point of application. Van Overbeek (1938) considered that the poor phloem connexions to lateral buds may account for the auxin-controlled diversion of nutrients from correlatively inhibited buds. In his recent review, Loomis (1953), also concluded that 'a theory of phloem development and food transport controlled by auxin seems best to fit the available data'. Particularly marked diversion of food materials is effected by developing fruits, which can 'channel the food supplies of the plant sometimes to the point of causing the death of the organism'. In the correlative inhibition of lateral buds, however, Went (1939) would attach more importance to the diversion of specific factors for bud and stem growth ('*phyllocalines*'). Skoog (1950) has expressed yet another point of view based on interactions between auxin and nutrients in the growth and organ formation of tobacco callus tissue (Skoog and Tsui, 1948, 1951). He now considers that the removal of the terminal bud makes nutrients available for translocation to other meristematic regions, 'where the nutrient level will be increased so as to require a higher threshold value of auxin for inhibition of growth'.

In conclusion, there can be little doubt, from a consideration of both the existing literature and the work described in the present paper, that direct inhibition by auxin plays little or no part in the correlative inhibition of lateral buds. Neither the 'indirect' nor the 'diversion' hypotheses of auxin inhibition can be eliminated on the basis of the evidence yet available. Further information on the process of translocation and on the nature of plant-growth inhibitors is required for the solution of the problem. It is not unlikely that growth correlation may result from a system of interactions in which the effects of growth promoting and inhibiting substances are influenced not only by their relative proportions, but also by the general nutrient level. The increase in the permeability of the protoplast effected by auxin (Guttenberg and Beythien, 1951) may play a part in the movement of nutrients to auxin-production centres.

ACKNOWLEDGEMENTS

I wish to thank Professor C. W. Wardlaw for his continued interest and encouragement. I am also indebted to Mr. E. Ashby, for the photographs in Fig. 1, and Dr. J. A. Bentley for the supply of 3-indolylacetonitrile.

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The Effects of Indole-3-Acetic Acid and 2:4-Dichlorophenoxyacetic Acid on the Growth Rate and Endogenous Rhythm of Intact *Avena* Coleoptiles

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Received 7 June 1955

SUMMARY

The rates of elongation of the coleoptiles of *Avena* seedlings, subjected to intermittent immersion in solutions of IAA or 2:4-D for various total periods, were determined from measurements of photographs taken every hour by infra-red radiation.

Immersion in 17.5 mg./l. IAA for 1-5 hours caused a large increase in the growth rate followed by a depression. When the seedlings were immersed in 8.75 mg./l. IAA for periods of 12 or 24 hours the depression was partially overcome so long as the treatment was continued. Absorption of additional IAA by the coleoptiles reduced their geotropic sensitivity.

Penetration of 2:4-D (sodium salt) into the coleoptiles was slower than that of IAA and the resulting stimulation of the growth rate was less, particularly in unbuffered solutions. After the treatment the growth rate declined slowly to about the normal value.

Results with coleoptiles were very similar to those previously obtained with rhizomes of *Aegopodium* and suggest that inhibition of growth following stimulation by IAA may be of general occurrence. Possible causes of the inhibition are discussed and a comparison is made between the results with intact coleoptiles and observations made by others on coleoptile sections.

Temporary immersion of the seedlings in auxin solutions depressed the rate of elongation of the primary leaf while it increased that of the coleoptile. It caused little disturbance of the endogenous rhythm induced by change from light to darkness. The suggestion that such rhythms can be explained in terms of variation in concentration of IAA-oxidase is not supported.

INTRODUCTION

As mentioned in the introduction to a paper by Ball and Dyke (1954), a series of experiments was commenced in order to determine whether certain effects on the growth rate, recorded by Ball (1953) when rhizomes of *Aegopodium podagraria* were immersed for short periods in solutions of indole-3-acetic acid (IAA) or 2:4-dichlorophenoxyacetic acid (2:4-D), would be observed when similar treatment was applied to intact *Avena* seedlings. The preliminary experiments led to the detection of an endogenous 24-hour rhythm in the growth rate of the coleoptiles, which was induced when the seedlings were transferred from red light to darkness. Consequently, further experimentation using growth substances was suspended while this rhythm was

investigated and the present paper deals with results obtained after the original series of experiments had been resumed.

METHODS

The methods used for growing the *Avena* seedlings and for recording the elongation of the coleoptiles by infra-red photography have already been described by Ball and Dyke (1954), but a slight modification was required to prevent the seedlings from moving when the plant chamber was flooded. This consisted in covering the roots with several thicknesses of wet filter-paper held down by a strip of sponge rubber retained in position by contact with the front glass of the chamber.

Complete immersion of rhizomes of *Aegopodium* in solutions of IAA for periods of 45 min. produced no apparent ill effects, as the rhizomes showed considerable elongation during these periods. But when seedlings of *Avena* were similarly immersed there was a marked fall in the growth rate during the period of immersion, although greatly increased growth was recorded during the next hour. Better aeration was provided by using intermittent immersion. In the arrangement finally adopted 2,000 ml. of the solution was contained in an aspirator bottle connected by rubber and metal tubing to the bottom of the plant chamber which had a capacity of 1,600 ml. The bottle was raised and lowered by an electric motor operating through reduction gearing. In this way the level of the liquid was made to rise and fall inside the plant chamber so that the seedlings were alternately submerged and aerated during the period of treatment, air being drawn into the chamber as the level of the liquid fell. The coleoptiles were completely out of the liquid for approximately one minute in every three. This method gave good results and in several experiments the intermittent immersion was continued for as long as 24 hours. When water alone was used there was a slight depression in the growth rate at first, but when the treatment was continued for a few hours the growth rate reverted to normal.

During the experiments the temperature was 21–24° C. The solutions were made up in glass-distilled water which had been allowed to come to this temperature. About 2 ml. of ethanol was used for dissolving the IAA just before the solution was required. In a replicate of one experiment (IV) in which solution of the IAA was effected by means of hot water there was no significant difference in the results.

EXPERIMENTAL RESULTS

Control experiments. The growth rate of coleoptiles of intact *Avena* seedlings which have been transferred from red light to darkness and kept at constant temperature varies not only with their age but also with the phase of the endogenous rhythm induced by the transfer. Hence the effects of treatment with growth substances can only be estimated by comparison with a control. In our earlier work we found that a well-defined rhythm was obtained in two experiments where the transfer from light to darkness was made at

the 56th hour from the time the grains were soaked. Three additional experiments were performed under the same conditions and the results of the 5 replicates are shown in Fig. 1. The curve drawn from the means of the 5 sets of values was used as a control and is represented in subsequent figures by a series of small circles

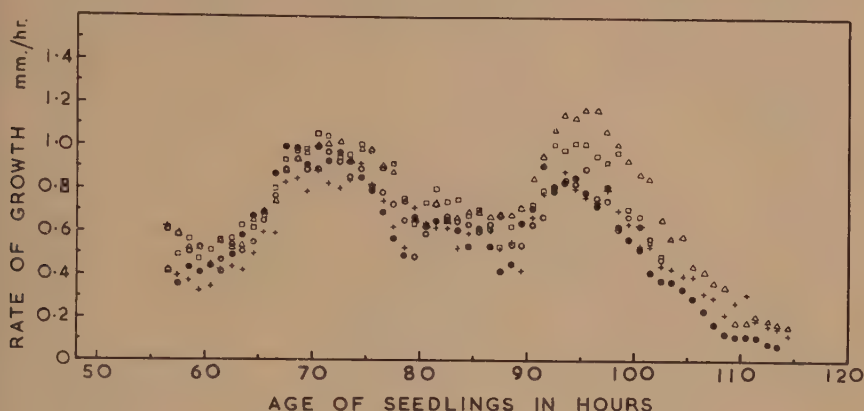


FIG. 1. Mean rates of extension growth of 20 coleoptiles in each of 5 similar experiments in which *Avena* seedlings were transferred from red light to darkness at the 56th hour from soaking. In subsequent figures the means of these 5 series are represented by small circles and are used as a control.

Effects of IAA. When rhizomes of *Aegopodium* were immersed in 10^{-4} M. IAA for 45 min. (Ball, 1953), the increase in the growth rate persisted for 5–6 hours, but when similar treatment—actually intermittent immersion for approximately one hour—was applied to *Avena* seedlings the effect disappeared much more quickly, as shown in Expt. I (Fig. 2). With a more concentrated solution (5×10^{-4} M.) the period of increased growth persisted longer (Expt. II), but the stronger solution tended to promote slight curvature of the coleoptiles. A longer period with the weaker solution was therefore adopted and Fig. 3 shows the results of treatment with 10^{-4} M. IAA (17.5 mg./l.) for a period of 5 hours applied at different phases of the rhythm.

As can be seen in these graphs immersion in IAA caused a rapid rise in the rate of elongation. After 2–3 hours the rate became relatively steady at a value two or three times that of the control. At the end of the treatment the rate declined rapidly, reaching the normal value in about 2 hours, and then fell further until 4–5 hours later it was only about half that of the control. This inhibition of growth following the increase corresponds very closely with what was observed by Ball (1953) when rhizomes of *Aegopodium* had been immersed in IAA. Its possible cause will be discussed later.

A point of considerable interest, in view of the hypothesis recently put forward by Galston and Dalberg (1954), is the extent to which treatment with IAA affects the endogenous rhythm of the coleoptiles. These authors consider that certain rhythmical movements in plants are due to rhythmicity of

auxin level. They further suggest that 'the adaptive formation and de-adaptive disappearance of IAA-oxidase, together with the existence of an IAA-producing centre in the plant, provide the essential features of a self-contained mechanism for production of rhythmicity in auxin level'.

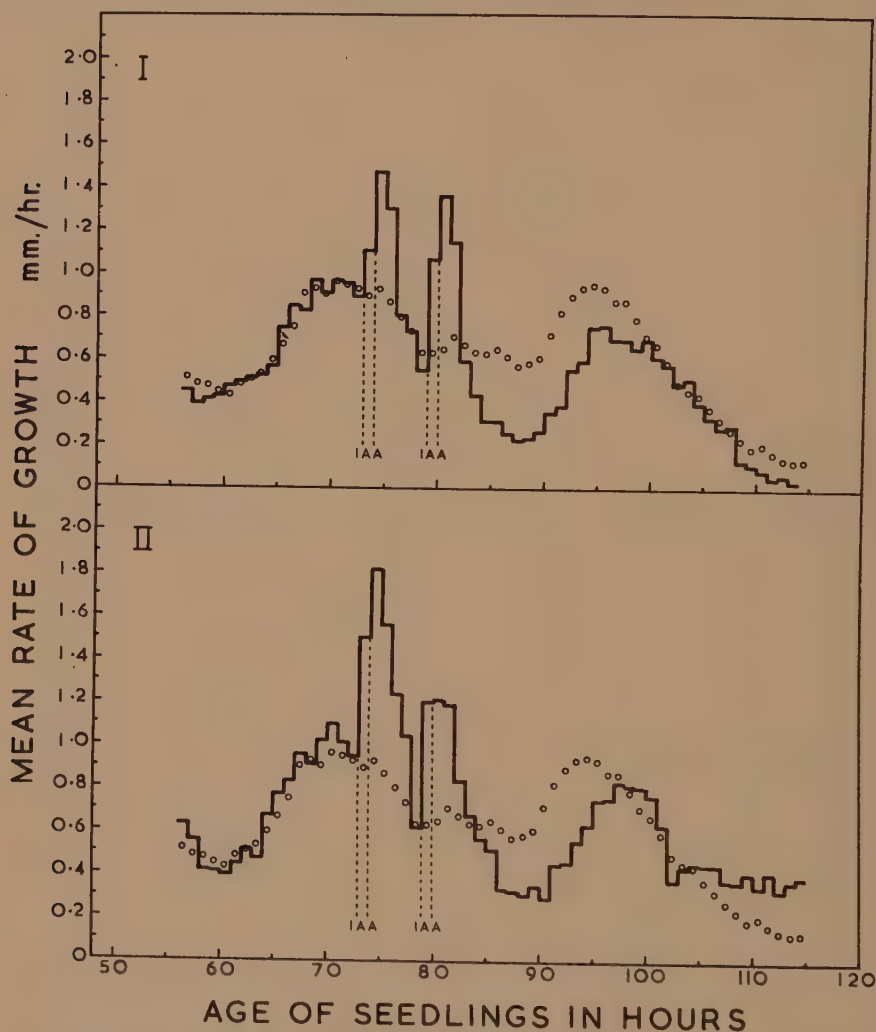


FIG. 2. Graphs I and II show the effects of one hour's intermittent immersion in IAA solution, at the 73rd hour and again at the 79th hour, on the growth rate of coleoptiles of seedlings transferred from red light to darkness at the 56th hour. In I the concentration of IAA was 10^{-4} M. (17.5 mg./l.) and in II it was 5×10^{-4} M. The control curve is represented by a series of small circles.

It is difficult to see how such a scheme could account for a periodicity which remains close to 24 hours independently of changes in temperature (Ball and Dyke, 1954), since the time taken to complete the cycle would depend amongst other things on the rates of formation and disappearance of the oxidase and on

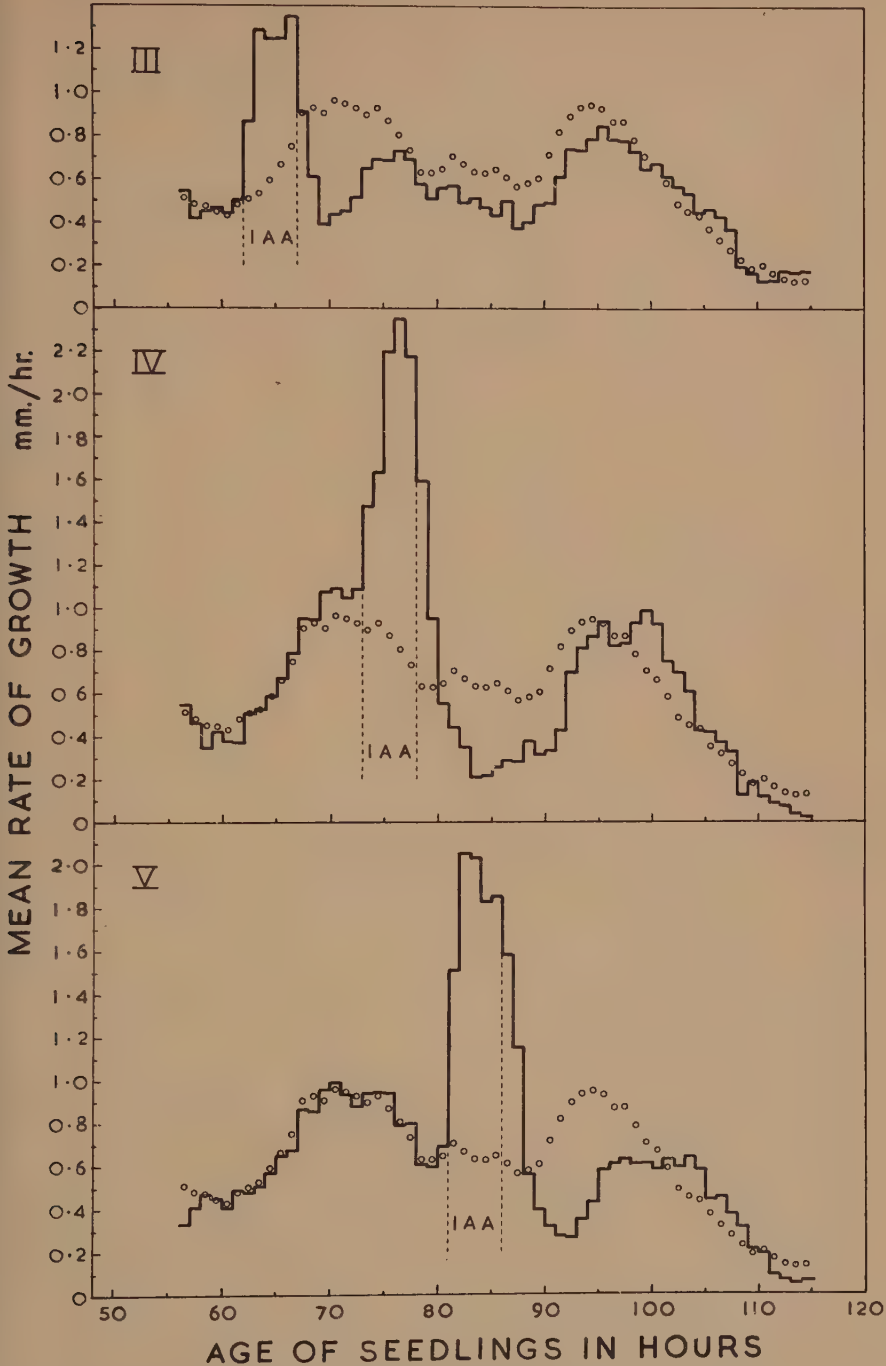


FIG. 3. Effects on the growth rate of intermittent immersion in 10^{-4} M. IAA for a 5-hour period at different phases of the endogenous rhythm. Treatment commenced at 62nd hour in III, at 73rd hour in IV, and at 81st hour in V.

its activity, all of which would presumably be affected by temperature. Moreover positive evidence against the hypothesis of Galston and Dalberg is afforded by the experiments recorded in Fig. 3. Although the increase in the growth rate induced by IAA and the subsequent depression do cause the growth-rate curves to diverge considerably from the control, the position of the second peak of the endogenous rhythm is only slightly affected by the timing of the auxin treatment. In Expt. V (Fig. 3) the IAA treatment was applied 8 hours later than in IV, but the difference in the timing of the second rhythmical peak in the two experiments is less than 3 hours. In Expt. V the second peak occurs only about 16 hours after the peak induced by the IAA treatment. In accordance with the hypothesis of Galston and Dalberg it would appear that the two peaks should be separated by approximately 24 hours.

It is not contended that the rhythmical rise and fall in the growth rate of the *Avena* coleoptile is not due to changes in auxin content, or that the auxin after it has been formed would not be subject to destruction by an adaptively produced IAA-oxidase. But the effects of this enzyme, if it is produced in this way, would seem to be secondary, the changes in auxin content being induced by some more fundamental time-keeping mechanism, the nature of which is still unknown. Since treatment with auxin solution had approximately the same effect if applied at a time when the normal growth rate was high as it had when it was low (cf. IV and V, Fig. 3), it is unlikely that the rhythmical variations in the growth rate are due to changes in the sensitivity of the tissues to IAA.

In experiments on *Aegopodium* it was found by Ball (1953) that the depression in the growth rate which appeared after short treatments with IAA could be partially overcome by prolonging the period for which the treatment was applied. Fig. 4 shows the results of similar experiments on *Avena* where the seedlings were exposed to intermittent immersion in solutions of IAA for periods of 12 and 24 hours. Owing to the tendency of these prolonged treatments to cause curvature, a lower concentration of IAA (5×10^{-5} M.) was used.

In both experiments (VI and VII) the growth rate rose to a maximum and then declined. (The decline was not due to disappearance of the IAA, since substitution of the original solution by a freshly prepared one had no effect.) The rate then became almost steady at a value which was maintained so long as the treatment was continued. It was considerably above that of the control. Hence the marked depression in the growth rate, recorded in the previous experiments following a short treatment with IAA, is not due to exhaustion of some essential food factor. At the end of the prolonged treatment the growth rate fell rapidly almost to zero, but where the treatment had lasted for only 12 hours there was a subsequent rise which may be regarded as the second peak of the endogenous rhythm.

Curvature of the coleoptiles as a result of immersion in solutions of IAA has already been mentioned. This curvature is of two kinds. There is first, a small but highly significant tendency for the vertical coleoptile to bend away from the side on which the grain is situated, the curvature reaching a maximum

about 4 hours after the commencement of the treatment. In 6 experiments in which measurements were made, 90 per cent. of the coleoptiles showed bending in this direction. The average amount was $8.6 \pm 0.77^\circ$. The cause of the

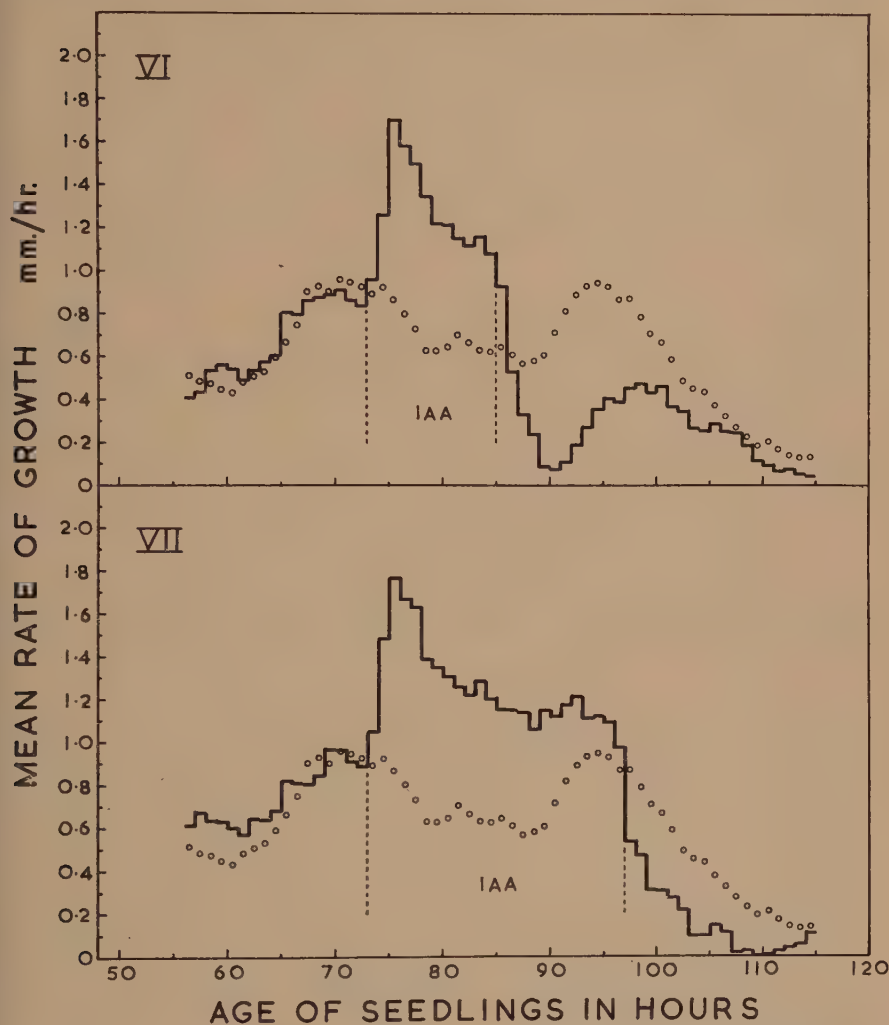


FIG. 4. Effects of intermittent immersion in 5×10^{-5} M. IAA for 12 hours (Graph VI) and 24 hours (Graph VII). Immersion commenced at 73rd hour in each case.

curvature was not determined, but it is worth noting that it was not apparent during the rise and fall in the growth rate in the control experiments, but only occurred when additional auxin was supplied.

The second type of curvature results from an excess of auxin causing increased plasticity at the same time as the geotropic sensitivity is reduced. Similar phenomena were recorded by Burkholder (1941) when agar blocks

containing large concentrations of auxin were applied to the cut surface of decapitated coleoptiles. Anker (1954) showed also that during the time non-decapitated coleoptiles were immersed in 5.7×10^{-5} M. IAA the negative geotropic curvature was much reduced. We carried out a few tests on intact seedlings stimulated in a horizontal position after they had absorbed additional

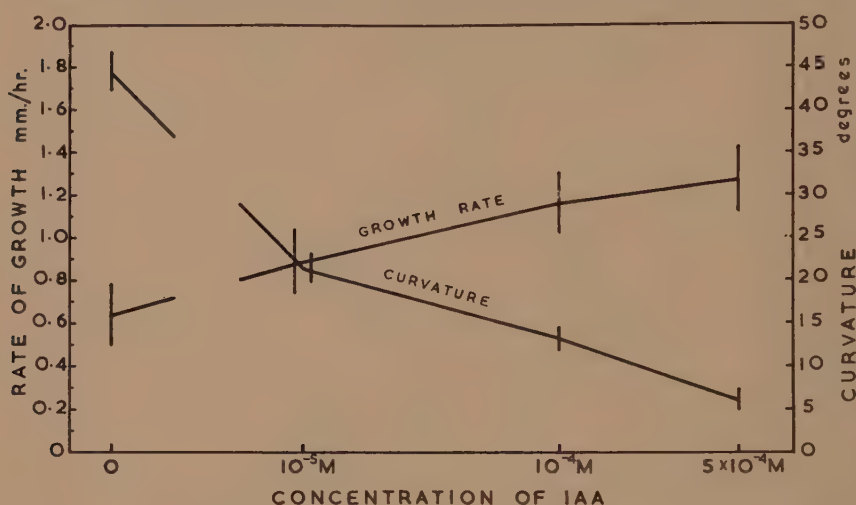


FIG. 5. Mean rates of growth and negative geotropic curvature of *Avena* coleoptiles, during 2 hours in the horizontal position, immediately following 4 hours intermittent immersion of seedlings in water, or in solutions of IAA of concentrations shown. Vertical lines represent twice standard error of means.

auxin. They were first subjected to intermittent immersion in water or solutions of IAA for 4 hours. At the end of this period the treatment was discontinued and the whole apparatus turned on its side so that the coleoptiles were approximately horizontal. The mean values for growth rate and upward curvature during the next 2 hours were subsequently determined from the hourly photographs and are shown in Fig. 5.

These curves indicate that with increasing amounts of auxin the geotropic response decreases, although at the same time the general rate of elongation becomes greater. Evidently, with more auxin present in the tissues, small differences in concentration induced by gravity on the two sides of the organ have less effect.

Owing to the decrease in geotropic sensitivity which occurred in most of our experiments, the normal tendency of the coleoptiles to grow vertically upwards was greatly reduced. Consequently, where the treatment with auxin was prolonged, many of them began to bend in various directions. Then the weight of the coleoptile in conjunction with the greater plasticity of the tissues induced by the auxin caused the curvature to increase. In addition there is the direct action of auxin in causing curvature as recorded by Ketellapper (1953) when coleoptile sections were floated on strong solutions of IAA. In our

experiments the main region of bending of many of the coleoptiles was just above the base and some of them bent down until they became almost horizontal. Although these continued to grow, their curvatures made accurate measurement of length difficult, or even impossible if they were no longer within the photographic field. For example, in the experiment where treatment with IAA was continued for 24 hours (VII), the later estimates of growth rate could only be based on 13 coleoptiles out of the original 20.

Where treatment with auxin is prolonged, or higher concentrations are used, the tendency of the coleoptiles to become curved diminishes the usefulness of the method described here for experimenting with whole seedlings. An improvement might be effected by confining each coleoptile within a narrow vertical channel, but contact with the sides of such a channel would be likely to affect the rate of growth.

Owing to difficulties arising from these curvatures prolonged treatments with relatively high concentrations of IAA were not attempted, but the effect of intermittent immersion for one hour in 10^{-3} M. IAA (175 mg./l.) was studied. The result (Expt. VIII, Fig. 6) was somewhat surprising, but it was fully confirmed in a replicate (IX). Comparison of these curves with those in Fig. 2 shows that increase in concentration of IAA from 10^{-4} to 10^{-3} M. has little effect on the maximum rate of growth, but does extend considerably the period during which an increased rate of elongation persists after one hour's treatment. With the high concentration of IAA the increase in the growth rate is followed by a short-lived depression in which the rate falls to a value about equal to or just below that of the control. Then follows a second peak which is very much higher than the control. The reason for this remarkable rise in the growth rate of the coleoptile is not definitely known, but it is likely that it is connected in some way with an inhibitory effect of the treatment on the rate of elongation of the enclosed primary leaf.

In experiments where the treatment with auxin was prolonged it was noticeable that the emergence of the primary leaf was much later than in the controls. Table I gives the age of the seedlings when 10 out of the 20 leaves had emerged in the control experiments and after various treatments with IAA commencing at the 73rd hour. In many of the photographs the position of the tip of the leaf before it emerged could be seen sufficiently clearly to enable measurements to be made. The Table shows the mean elongation of as many leaves in these experiments as could be measured accurately over a 24-hour period starting from the commencement of the treatment. The mean total elongation of the corresponding coleoptiles is included for comparison, but in most of the experiments the rate of elongation of the coleoptiles varied considerably during the 24-hour period, as shown in the graphs.

It is obvious from the data in Table I that although treatment of the seedling with IAA accelerates the growth of the coleoptile it depresses the rate of elongation of the enclosed leaf. Both processes play a part in delaying the emergence of the leaf tip. These effects tend to increase as the period of treatment is prolonged. They are also very marked after one hour's intermittent

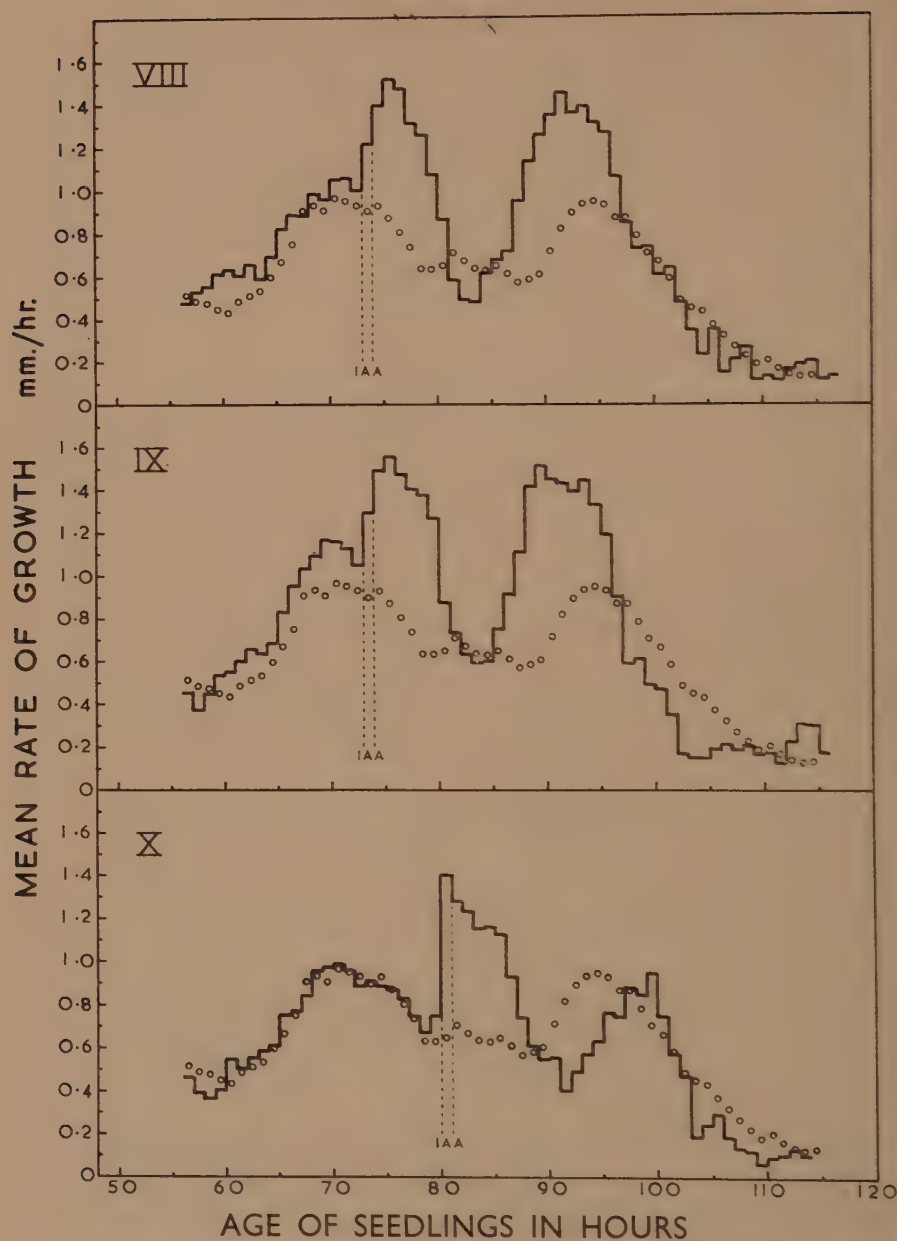


FIG. 6. Graphs VIII and IX represent replicate experiments and show the effects of 1 hour's intermittent immersion in 10^{-3} M. (175 mg./l.) IAA at the 73rd hour. In X the immersion in 10^{-3} M. IAA was at the 80th hour.

TABLE I

Effects of Intermittent Immersion in IAA on the Elongation and Emergence of the Primary Leaf

Expt.	Treatment		Age in hours when 10th leaf emerged	Elongation in mm. 73-97 hours	
	Conc. of IAA mg./l.	Period in hours		Leaf	Coleoptile
Control	—	—	95±2.7	24.26±0.58	17.99±0.60
IV	17.5	73-78	109	18.48±0.79	19.22±0.48
VI	8.75	73-85	108	15.92±0.70	19.36±0.69
VII	"	73-97	133	14.26±1.86	32.54±1.63
VIII	175.0	73-74	> 121*	11.14±0.44	23.53±1.24
IX	"	"	167	12.64±0.54	27.89±0.59

* In Expt. VIII no leaves had emerged when recording ceased at 121st hour.

immersion in a relatively high concentration of IAA (175 mg./l.), the subsequent elongation of the leaf being only about half that of the control, while the elongation of the coleoptile is about 50 per cent. greater. A tentative explanation of the second large increase in the growth rate of the coleoptile, which occurred in these experiments (VIII and IX), is that the depressed growth of the primary leaf makes available to the coleoptile an additional supply of some growth-promoting factor. As a result, the influence of the endogenous rhythm in increasing the growth rate becomes effective sooner and operates with greater intensity than in the controls. In many cases the apical region of the coleoptile extending beyond the leaf tip began at this stage to show erratic curvature indicating that it possessed an excess of auxin. If this conclusion is correct, the excess of auxin is unlikely to have persisted from the original treatment and must have been newly produced.

Expt. X (Fig. 6) was similar to VIII and IX except that the immersion in 10^{-3} M. IAA took place 7 hours later at the 80th hour. Comparison of the curves shows that the first effect of the treatment in X is like that in VIII and IX, but the depression now coincides with the time when the second peak of the endogenous rhythm is due to commence. Consequently the rise in the growth rate is delayed and is smaller than in the previous two experiments. Emergence of the 10th leaf was at the 135th hour.

Effects of 2:4-D. Working with rhizomes of *Aegopodium* a marked difference was found by Ball (1953) in the effects of short immersions in solutions of IAA and 2:4-D. Both substances caused stimulation of the growth rate, but differed in their after-effects. With IAA stimulation was followed by a sudden fall in the growth rate which dropped temporarily to one third or less of its original value; with 2:4-D the decline was gradual and the rate fell only to about the level which prevailed before the treatment. It was therefore of considerable interest to determine whether a similar difference would be observed when *Avena* coleoptiles were used.

The intact coleoptiles proved less responsive to 2:4-D than they did to IAA. The resistance to 2:4-D of cereals compared with many dicotyledonous

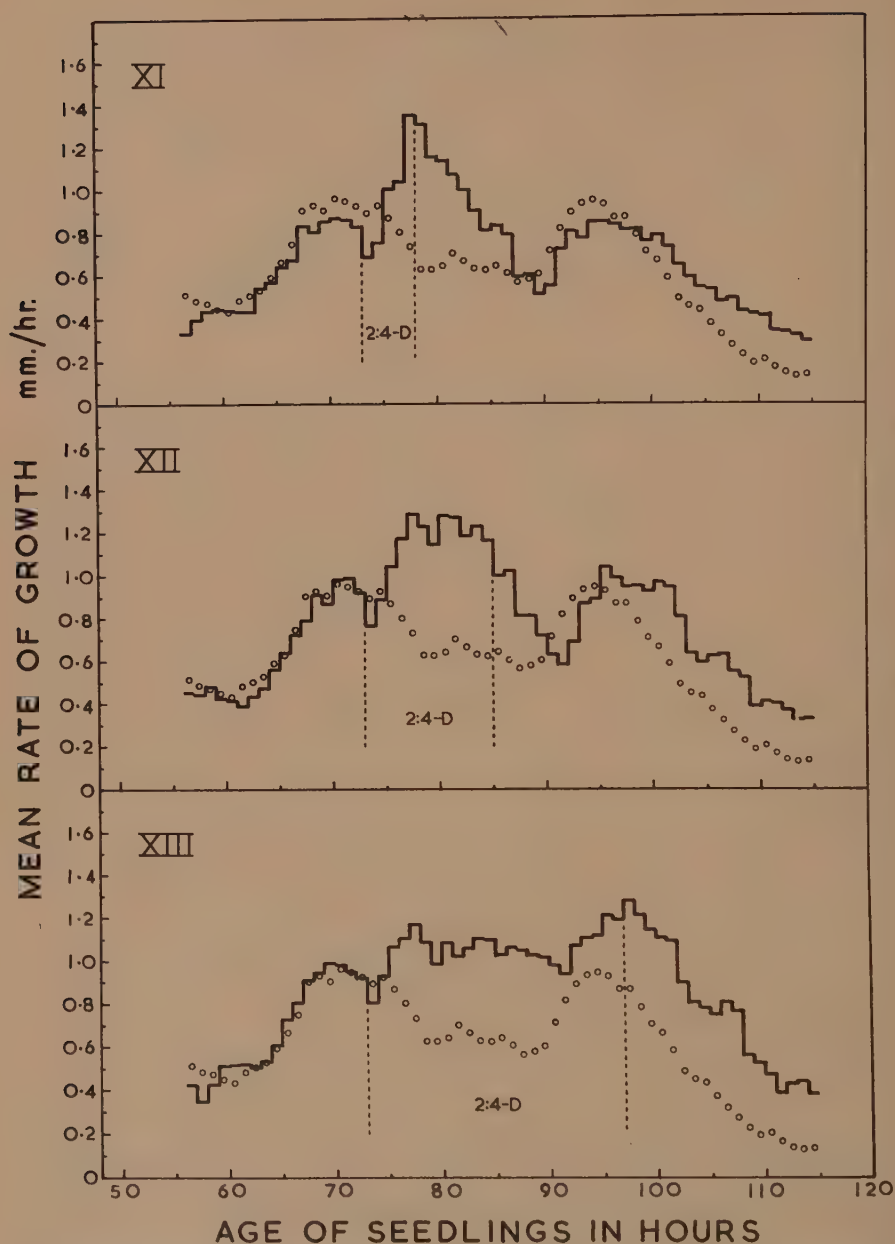


FIG. 7. Graph XI shows the effects of intermittent immersion in 10^{-4} M. 2:4-D (sodium salt) for a 5-hour period. In XII the seedlings were immersed in 5×10^{-5} M. 2:4-D for 12 hours, and in XIII for 24 hours. In each case the treatment commenced at the 73rd hour.

plants has been known for some time. More recently, it has been shown by Gallup and Gustafson (1952) that 2:4-dichloro-5-iodophenoxyacetic acid, and by Doxey (1953) that 2-iodo-4-chlorophenoxyacetic acid, both labelled with I^{131} , were absorbed much more slowly by leaves of oats than by leaves of broad bean. Translocation within the oat leaves was also much slower.

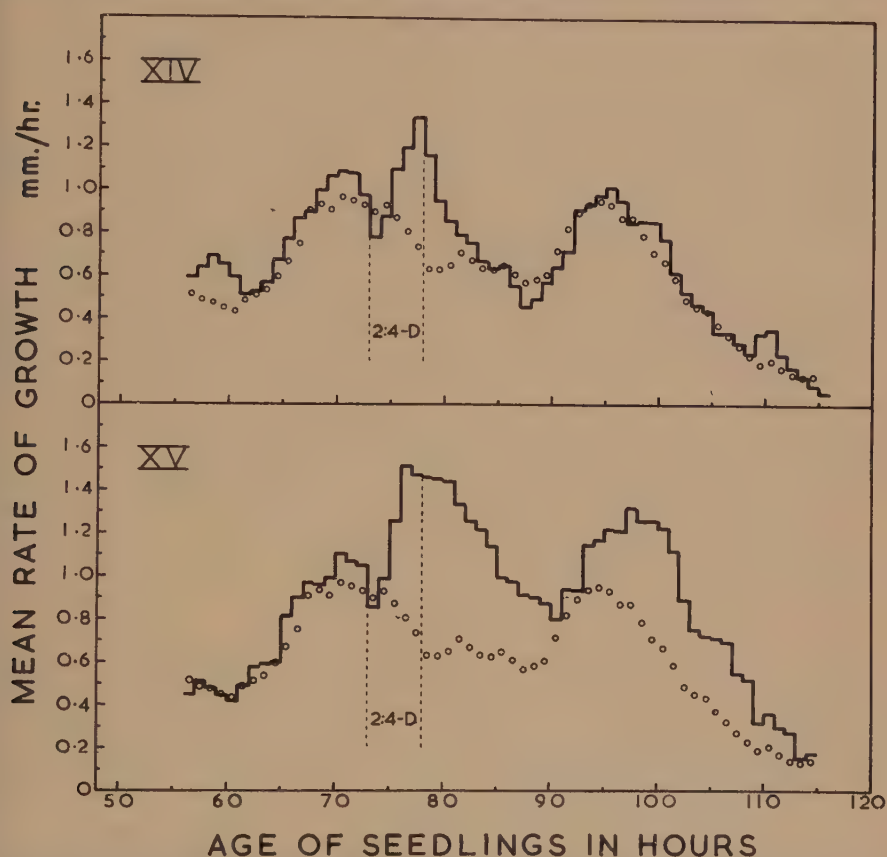


FIG. 8. In XIV the seedlings were subjected to 5 hours intermittent immersion in 5×10^{-4} M. 2:4-D at the 73rd hour. The treatment in XV was similar to that given in XI, but 2:4-D was dissolved in phosphate buffer at pH 5.4.

In our experiments the technique employed was identical with that already described, except that various concentrations of 2:4-D (sodium salt dissolved in distilled water) were used in place of IAA. 10^{-5} M. solutions caused very little response, but Fig. 7 shows the effects of intermittent immersion in 10^{-4} M. solutions for 5 hours (XI), and in 5×10^{-5} M. solutions for periods of 12 and 24 hours (XII and XIII). Slow penetration is evidenced by the fact that the maximum growth rate is not reached until about 5 hours after the commencement of the treatment. As in the experiments on rhizomes there is a slow decline after the treatment, with little or no indication of an inhibitory

effect. Where the treatment was prolonged there was a considerable amount of erratic curvature, as occurred in similar experiments with IAA. Also, as in the experiments where IAA was used, prolonged treatment depressed the elongation of the primary leaf and delayed its emergence from the coleoptile. In the 3 curves shown in Fig. 7, and the two in Fig. 8, the second peak of the endogenous rhythm is clearly evident. There is a tendency for this peak to appear somewhat later than in the control, the delay amounting at most to about 3 hours. A similar retardation occurred in many of the experiments with IAA.

Attempts were made to increase the response to 2:4-D. Firstly, a higher concentration (5×10^{-4} M.) was used. The resulting curve (XIV, Fig. 8) shows that the response was no greater than with the 10^{-4} M. solution (XI, Fig. 7) and that the effect disappeared earlier. With a still stronger solution (10^{-3} M.) there was evidence that inhibition quickly followed stimulation, but excessive curving of the coleoptiles made accurate measurement almost impossible and the results are not reproduced here. Lastly, in Expt. XV the 2:4-D was dissolved in a 0.015 M. potassium phosphate buffer at pH 5.4, but in other respects the experiment was similar to XI (Fig. 7). The growth-rate curve rises somewhat higher and continues at a higher level after the treatment than in XI, but the general pattern of the two curves is otherwise much the same. No further experiments on the effect of pH on the response of *Avena* seedlings to 2:4-D were undertaken, but in a similar experiment in which they were immersed in distilled water containing the phosphate buffer only, no stimulation was observed.

DISCUSSION

The general similarity between the responses of intact seedlings of *Avena* and of rhizomes of *Aegopodium* to temporary immersion in solutions of IAA and 2:4-D has already been pointed out. This similarity shows that the phenomena observed are not confined to the rather specialized rhizomes and suggests that they may be of general occurrence.

One of the more interesting problems is the cause of the depression in the growth rate which follows stimulation by means of IAA. Since this depression can be overcome to a large extent by repeating or prolonging the treatment with IAA it cannot be ascribed to shortage of any essential food material. In connexion with the work on *Aegopodium* the suggestion was made by Ball (1953) that the depression was due to an inhibitor produced either directly from IAA or from some other source as a result of the action of IAA. Kefford (1953) using chromatography obtained some indication of an increase in the amount of an inhibitor in rhizomes treated with IAA. On the other hand, Bennet-Clark and Kefford (1954) have shown that when coleoptile sections are grown in 50 mg./l. or 30 mg./l. of IAA there is no outward diffusion of inhibiting substances into the external solution.

Recently an alternative possibility has been disclosed by the claim of Galston and Dalberg (1954) that IAA-oxidase is an adaptive enzyme whose formation

in tissue occurs as a response to the presence of auxin. If this is so, then the depression which follows the increased rate of growth due to treatment of rhizomes or coleoptiles with IAA could be attributed to actual destruction of this substance in the tissues. The IAA destroyed would include not only that which had entered the tissue from the solution, but also any produced by the plant itself. Hence the rate of elongation would fall below that of the control. Where immersion in solutions of IAA is continued for more than a few hours, a relatively high concentration of this substance would first of all develop in the tissues causing a high growth rate. Then, as IAA-oxidase began to accumulate, increased destruction of IAA would occur and the growth rate would fall. But, provided fresh supplies of IAA continued to enter the tissues from the external solution, the fall in concentration of IAA would continue only until a level was reached where the rate of entry just balanced the rate of destruction. As soon as external supplies had been cut off, the high concentration of IAA-oxidase would cause a rapid destruction of the remaining IAA and the growth rate would fall rapidly to a low level. Disappearance of the enzyme would then allow the building up of fresh amounts of IAA in the tissues, provided their metabolism was still active. The newly formed auxin would then cause renewed growth. Such an explanation fits in very well with the results of the 12- and 24-hour treatments shown in Fig. 4. It must, however, be pointed out that they can equally well be explained on the supposition that the presence of IAA in the tissues in large concentrations leads to the formation of an inhibitor.

Galston and Dalberg (1954) state that the maximum induction of IAA-oxidase in pea seedlings occurs when the concentration of IAA in the external solution is about 10^{-7} M., while concentrations in excess of about 10^{-6} M. produced marked inhibition of the activity of the enzyme. If the same holds good for *Avena* coleoptiles, then the solutions used in our experiments, which had concentrations varying from 5×10^{-5} to 10^{-3} M., not only would have failed to promote the production of IAA-oxidase, but would actually have diminished its activity. If this is the case, the depression in the growth rate cannot be attributed to the action of IAA-oxidase but must have been due to the production of an inhibitor.

Turning now to the results obtained with 2:4-D. After the treatment the decline in the rate of growth is very gradual and there is little or no tendency for the rate to drop below that of the control. Galston and Dalberg state that 2:4-D is as effective as IAA in inducing the formation of IAA-oxidase. But this enzyme is specific for IAA and would have no effect on 2:4-D. Hence there would be no depression of the growth rate below that of the control so long as the 2:4-D persisted after the treatment and masked the effect of destruction by the enzyme of IAA produced by the plant. However, the fact that a fully developed peak in the growth-rate curve, apparently due to the endogenous rhythm, appears soon after, or even during the treatment with 2:4-D (cf. Expt. XIII, Fig. 7), makes it doubtful whether induction of IAA-oxidase by the 2:4-D did actually occur.

Comparison of the results of the present series of experiments with those obtained by other workers on coleoptile sections is only justified where the extension of the sections has been followed at short intervals of time and where the concentrations of IAA used are of the same order. This is the case with some of the experiments described by Bennet-Clark and Kefford (1954). For example, their experiment in which 3 mm. coleoptile sections were floated on 10.0 mg./l. IAA and the extension rate plotted at approximately 3-hourly intervals is closely comparable to those in which *Avena* seedlings were subjected to intermittent immersion in 5×10^{-5} M. (8.75 mg./l.) IAA for 12 or 24 hours. In both sections and intact coleoptiles the rate of elongation is high for about the first 5 hours and then drops to a lower level where it remains approximately steady. As already pointed out, such behaviour can be explained either in terms of the development of a growth inhibitor, or of an enzyme system which causes destruction of the auxin. On the other hand, Foster, McRae, and Bonner (1952) working with a lower concentration, 1.0 mg./l., in presence of a potassium maleate pH 4.5 buffer, found a linear relationship between section growth and time over a period of 18 hours. Bennet-Clark and Kefford also found that with low concentrations, up to and probably above 0.7 mg./l., growth was linear with time. Under such conditions the inhibiting factor, whatever its nature, apparently does develop. Further evidence on this point could probably be obtained more conveniently by working with coleoptile sections rather than intact seedlings, particularly as the technique we have employed allows only one treatment to be studied at a time. The use of an auxanometer, such as that described by Ranson and Harrison (1955), while affording greater accuracy in actual measurement, would be even more time-consuming, as it can only deal with single coleoptiles, and a considerable amount of replication would be necessary.

It has already been pointed out that temporary immersion of *Avena* seedlings in solutions of IAA or 2:4-D has little permanent effect on the endogenous rhythm of the coleoptiles. In all experiments recorded here, except Expt. III, the immersion was commenced after the first peak of the rhythm. In every experiment, except VII (Fig. 4), where the period of immersion in IAA extended beyond the time when the second peak became due, the occurrence of this peak is clearly indicated in the graphs. Where it became due at a time when factors inhibiting growth were in operation, it tended to be a few hours late. If, on the other hand, growth promoting factors induced by the treatment were operating (Expts. VIII and IX), the peak was a few hours early. It seems therefore that the treatment applied to the seedlings affects merely the manifestation of the endogenous rhythm, probably by increasing or decreasing the internal auxin concentration, and that the actual time-keeping mechanism remains undisturbed.

We wish to express once again our thanks to Professor T. A. Bennet-Clark, F.R.S., for the interest he has taken in this investigation and for kindly providing the necessary facilities. The work was carried out with the aid of a grant from the Agricultural Research Council.

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The Translocation of Antibiotics in Higher Plants

II. THE MOVEMENT OF GRISEOFULVIN IN BROAD BEAN AND TOMATO

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Received 16 May 1955

SUMMARY

The uptake and translocation of griseofulvin from water culture by broad bean and tomato has been studied; observations were also made on its decay in broad bean. In most cases griseofulvin was determined by bioassay. Where possible the bioassay was checked by chemical estimations and was found to be adequate.

The amount of griseofulvin taken up by the broad bean was proportional to the volume of water transpired for any single concentration of the treating solution and the decay was exponentially related to the time of exposure in the tissues. The accumulation of griseofulvin in the tissue had, therefore, an exponential component but within the limits of error there was constant relationship between accumulation and transpiration over the time periods used in the trials. The rate of accumulation in tomato was also constant. The amount of griseofulvin accumulated by both bean and tomato after a definite time was a linear function of the concentration of the treating solution.

There were two processes involved in the uptake of griseofulvin by the broad bean: (a) an initial rapid entry into the roots which was inhibited by sodium azide and dinitrophenol at concentrations which did not reduce transpiration; and (b) a prolonged uptake linearly related to transpiration which was not affected by these concentrations of the inhibitors.

INTRODUCTION

THERE is much evidence that griseofulvin is taken up and translocated by a number of higher plants. It has been found in the guttation fluid, in water extract of shoot tissue, and in sap expressed from plants grown in solutions of the antibiotic (Brian, Wright, Stubbs, and Way, 1951; Stokes, 1954; Crowdy and Pramer, 1955). Griseofulvin appears to be translocated without chemical change and has been recovered in pure form from the shoots of root-treated broad bean plants (Crowdy, Gardner, Grove, and Pramer, 1955). Griseofulvin is a highly effective systemic fungicide (Brian *et al.*, 1951; Stubbs, 1952). This gives a study of its translocation particular interest.

Preliminary studies on the translocation of griseofulvin were made by Brian and his colleagues (1951) who showed that the antibiotic could be detected in the guttation fluid of oat seedlings 7 days after treatment and that plants receiving an initial 15-day treatment continued to excrete griseofulvin for periods of 3 to 4 weeks. Stokes (1954) made a more extensive study of the factors influencing the appearance of griseofulvin in the guttation fluid of wheat seedlings grown in solutions of the antibiotic. She found that there was a minimum lag of at least 2 days between the start of treatment and the appearance of measurable quantities of griseofulvin in the guttation fluid and that the lag period was somewhat longer when the concentration of the treating solution was low. After this lag period, the concentration of antibiotic in the guttation drops increased with the concentration of the solution used for treatment, the temperature, the rate of transpiration of the plant, the period of treatment and the interval between samples. It appeared that factors which increased transpiration increased also the rate of griseofulvin movement and that there was some accumulation of the antibiotic. The present investigations amplify Stokes's results.

MATERIALS AND METHODS

In the experiments described below two varieties of broad bean (*Vicia faba* L.) 'Express' and 'Sutton's Dwarf' and one of tomato (*Lycopersicum esculentum* Mill.) 'Kondine Red' were used. The conditions in which the plants were raised and treated have already been described (Crowdy *et al.*, 1955). The beakers containing plants and solutions were weighed at the beginning and end of each experiment and the weight of solution added during an experiment was noted: these figures gave a measure of the transpiration of the plants. In making up the treating solutions, appropriate volumes of a 1 per cent. solution of griseofulvin in acetone were added to the growing medium. Preliminary data (Bartels-Keith, unpublished results) suggest that in pure water the solubility of griseofulvin is about 9 $\mu\text{g.}/\text{ml.}$ at room temperature and the higher concentrations used were probably supersaturated.

The griseofulvin in the treated plants was estimated by bioassay, in expressed sap as described by Crowdy and Pramer (1955) and in chloroform extracts of the plant tissues. Before extraction the roots were washed in 4 changes of water. The last washing contained negligible quantities of griseofulvin showing that practically all the readily removed antibiotic had been washed off.

Extraction and bioassay

The following procedure was used for the chloroform extraction of the plants. Samples of tissue weighing between 5 g. and 20 g. were macerated in a high-speed macerator in chloroform and the macerate was extracted at room temperature for 2 or 3 days. The residue from the cold extraction was then extracted for 30 minutes in a Soxhlet apparatus. The chloroform solution was evaporated to dryness and the green residue dissolved in a mixture of 3 ml. water and 6 ml. light petroleum (b.p. 40–60° C.). This mixture was allowed to

separate into two layers of which the petroleum contained most of the oils and green colouring matter. The aqueous layer was withdrawn and the excess petroleum dissolved in it removed by evaporation under reduced pressure at room temperature. The aqueous layer was then ready for bioassay. The preparation of expressed sap has already been described (Crowdy and Pramer, 1955). The bioassay procedure has been described by Brian, Curtis, and Hemming (1946). Conidia of *Botrytis allii* were incubated for 17 hours at 25° C. in serial dilutions of half strength Weindling solution containing the extracted griseofulvin and the number of dilutions required to eliminate the waving effect characteristic of griseofulvin, the dilution end point, was noted. Each step in the series of dilutions contained half the concentration of extract present in the preceding step.

Calibration of extraction technique

The losses of griseofulvin during extraction were appreciable and the statistical relationship between the griseofulvin assayed after extraction and that initially present in the tissues was determined by extracting tissues to which a known weight of griseofulvin had been added. In a typical experiment 100, 50, 25, and 12.5 $\mu\text{g.}$ of griseofulvin were added to the macerated tissues immediately prior to extraction and the extractions were carried out in quadruplicate. The dilution end points, e , obtained from these extractions were compared with the end points which would have been expected had the extraction procedure been perfect, E . These two quantities were found to be linearly related

$$e = m.E + n. \quad (a)$$

In this assay, in which the stages were 1:2 dilutions, there is a logarithmic relationship between the concentration of griseofulvin actually present in the sample and the dilution end point recorded. This relationship was used to determine the weight of griseofulvin, G , dissolved in a standard volume of water, v

$$G/v = c.2^E \quad (b)$$

where E is the dilution end point. The constant, c , was determined experimentally and for the present series of experiments was 0.39 $\mu\text{g./ml.}$ units. Owing to losses during extraction the recorded dilution end point, e , underestimated the amount of griseofulvin originally present in the tissues and the corrected value, E , calculated from equation (a), was used in the solution of equation (b).

The extraction procedure was calibrated for the various tissues used in the experiments. In most cases the calibration was repeated and the results did not differ significantly in the two trials. The equations relating the observed dilution end points, e , with the maximum possible end point, E , are shown in Table I.

There were no significant differences either in slope or position between the equations for the parts of the plants which have been grouped together with the same equation. There was a significant difference in slope between the line

derived from the leaves and stems of the Express broad bean and the remainder of the lines, which had the same slope but differed significantly in position.

TABLE I

Calibration of Extraction: Relation between Observed End Point, e, and Theoretical Maximum End Point, E.

Tissue extracted	Equation
Broad bean, Express, leaves and stems . . .	$e = 0.65E - 0.315$
" " Dwarf, leaves . . .	$e = 1.0E - 1.7$
" " " stems . . .	$e = 1.0E - 0.7$
" " roots (both vars.) . . .	$e = 1.05E - 2.59$
Tomato, leaves, stems, and roots . . .	$e = 1.09E - 3.43$

An assay procedure which is based on a series of 1:2 dilutions can only detect differences of the order of 100 per cent. between individual samples and in most cases this order of sensitivity has not been reduced by extraction. Clearly the errors in estimation are very high but they can be evaluated in the statistical analysis of replicated experiments.

The accuracy of the assay.

In two cases it has been possible to compare the estimates of griseofulvin present in the tissues arrived at by the bioassay procedures described above with the weight of griseofulvin separated chemically from the tissue constituents and determined spectrophotometrically (Crowdy *et al.* 1955). In these cases broad beans were treated 3 days in solutions of 25 µg./ml. of griseofulvin, the shoots were macerated and extracted in chloroform and the extract, in chloroform, was divided into two parts one of which was prepared for chemical and the other for biological assay. The results of these trials are presented in Table II.

TABLE II

Comparison of Chemical and Biological Assays of Griseofulvin

Broad Bean variety	Vol. extract in chloroform assayed		Chemical assay		Bioassay		
	Chemically	Biologically	Total mg.	Conc. in original solution µg./ml.	Total µg.	Conc. in original solution µg./ml.	Dilution end point e
*Express	490	10	12	24	113	11	5
Dwarf	196	4	4.5	23	123†	31	7

* Experiment quoted by Crowdy *et al.* (1955) p. 372.

† An arbitrary correction, assuming leaves and stems contribute equally to the final total, has been made for the presence of both leaf and stem extracts in this sample.

In the case of the Express broad beans a dilution end point of 6 would have yielded a concentration in the original solution of 33 µg./ml. and for the Dwarf

beans an end point of 6 would have been equivalent to a concentration of 16 $\mu\text{g./ml.}$ Thus in both these cases the bioassay estimate was as close to that made by chemical methods as the limitations of the technique allowed.

EXPERIMENTAL RESULTS

The movement of griseofulvin in broad bean and tomato can be conveniently considered in stages; uptake, decay, and distribution within the plants. Data on these phases have been obtained by assaying the griseofulvin content of plants on solutions of different concentrations for various time intervals. It will be shown later that there is some decay of griseofulvin in the tissues; the rate of uptake will, therefore, be greater than the rate observed by assaying griseofulvin in the tissues; the latter will be referred to as the rate of accumulation. It is not necessarily implied that this is an active process. The water uptake of healthy plants was found to be a linear function of time in the experimental conditions and marked deviation from this relationship was taken to indicate phytotoxicity. It was, thus, not possible to distinguish between the effect of time and transpiration on the uptake of griseofulvin in the experiments conducted in controlled conditions.

Uptake by roots and movement to shoots.

The stages of uptake are illustrated in Tables III and IV. In the experiment illustrated in Table III whole plants and detached roots were treated in a solution of 25 $\mu\text{g./ml.}$ of griseofulvin. The roots and shoots were sampled separately at varying times after the beginning of treatment. Half the beakers in each treatment were aerated. Aeration had no effect on uptake and this treatment has been ignored in presenting the data. The means in the table were each derived from 6 replicates.

TABLE III
*Uptake of Griseofulvin by Whole Plants and Detached
Roots of Broad Bean*

Time in hours	Concentration of griseofulvin, $\mu\text{g./g.}$ (fresh wt.)		
	Whole plants		Detached roots
	Shoots	Roots	
0.5	2	32	25
1.0	4	24	35
3.0	8	29	50
6.0	19	34	27

Least significant difference is 16 ($P=0.05$)

This table shows that there was a rapid initial uptake of griseofulvin which was completed in 30 minutes, when the concentration in the roots approximated to that in the treating solution. The accumulation of griseofulvin in the

shoots in $\mu\text{g./g.}$ (fresh wt.), w , was linearly related to the period of treatment, t hours, and was described by the equation

$$w = 2.9t + 0.42.$$

Over the 6-hour period there was no clear sign of accumulation of griseofulvin in the roots although this could be demonstrated over longer time periods. A more extensive experiment of the same type is illustrated in Table IV. Four concentrations of griseofulvin were used for treatment and the maximum treatment time was extended from 6 hours to 48 hours; the roots and shoots of the whole plants were again sampled separately. The detached roots sampled after 48 hours were moribund and were rejected.

TABLE IV
*Uptake of Griseofulvin by the Roots of Whole Plants
and Detached Roots of Broad Bean*

Treatment time hr.	Conc. in roots, $\mu\text{g./g.}$ (fresh wt.)							Linear regression conc. in roots on time $\mu\text{g./g./hr.}$	
	Whole plants				Detached roots			Whole plants	Detached roots
	1	8	24	48	1	8	24		
Conc. of treating solution, $\mu\text{g./ml.}$									
25	21	15	30	76	27	44	95	1.3*	3.0*
12	13	17	31	49	8	25	34	0.8*	1.0*
6	5	10	10	18	12	27	32	0.2*	0.8
3	4	9	11	10	5	8	24	0.1	0.9
Linear regression conc. in roots on treat. conc.	0.8*	0.3	1.0*	3.1*	0.9	1.4*	3.2*	—	—

* Regressions significant and not deviating significantly from linear

As in the previous experiment there was a rapid initial uptake of griseofulvin by the roots until the concentration approximated to that of the treating solution; subsequently there was a significant linear increase with time. This increase was more rapid both in the more concentrated treating solutions and in the detached roots. This would be expected since a proportion of the griseofulvin entering the roots of whole plants is translocated to the shoots. The recorded increase in the concentration in the roots of whole plants represents the difference between the griseofulvin taken up and that translocated.

In this experiment the rate at which griseofulvin accumulated in the tissues as a whole was also a linear function of the treatment time and the rate of accumulation varied with the concentration of the treating solution. These lines are illustrated in Fig. 1 which also includes points showing estimated concentrations calculated by a method discussed later (p. 11).

The rates of accumulation shown in Fig. 1 were based on the amount of griseofulvin actually present in the tissues. If breakdown was appreciable, they would not represent the true rates of uptake. Information about these was

provided by chemical estimations in which a known weight of griseofulvin was supplied to the plants and the amount remaining in the treating solution at the end of treatment was measured. The actual amount taken up was found by difference. The results of four of these trials are presented in Table V. The initial concentration of the treating solution was 25 $\mu\text{g./ml.}$ in each case. The initial weight taken up has been calculated on the assumption that the roots

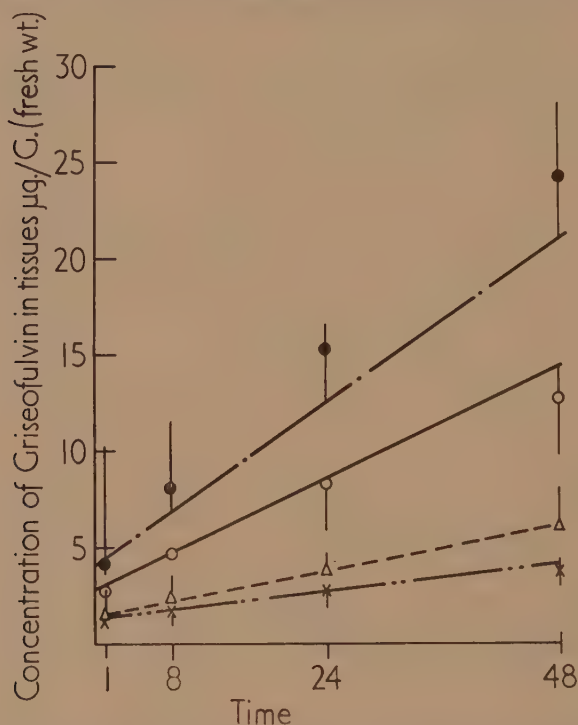


FIG. 1. Relation between treatment time, given in hours, and griseofulvin concentration in the tissues of broad bean treated with various concentrations of griseofulvin concentrations.

Treatment	Calculated	Observed
25 $\mu\text{g/ml}$:	●	— · —
12 $\mu\text{g/ml}$:	○	— — —
6 $\mu\text{g/ml}$:	△	- - - - -
3 $\mu\text{g/ml}$:	×	- · - · -

Deviations from experimental line allowed for $P = 0.05$.

attain the same concentration as the treating solution very soon after immersion and the rate of uptake has been calculated from the difference between the total griseofulvin taken and the weight allocated to the initial uptake. There is less variation between experiments when the rate of uptake is calculated from the water uptake suggesting that transpiration determined the uptake of griseofulvin. This would not be unexpected since transpiration seemed likely to be the main vehicle for transporting the antibiotic to the shoot. The rate of

transpiration would determine the deficit in the roots and, therefore, the rate of entry into the roots.

TABLE V

Rate of Uptake of Griseofulvin by Broad Bean Plants Treated with 25 µg./ml. Solution. Derived from Chemical Estimates

Expt. no.	Roots fresh wt. g.	Time days	Griseofulvin wts. mg.		Water transpired litres	Rates of uptake	
			initial uptake	subsequent uptake		mg./day	µg./ml. water
1	347	3	9	31	2.10	10.3	14.7
2	361	3	9	32	1.79	10.7	18.2
3	282	3.5	7	19	1.64	5.4	16.7
4	303	2	8	43	2.01	21.5	21.4

Decay of griseofulvin in broad bean plants

In experiments to determine the decay of griseofulvin in tissues, the plants were treated for an initial period and were then transferred to water. Water uptake was recorded during the treatment period. When the griseofulvin was to be estimated by bioassay the plants were sampled at the end of treatment and at varying intervals thereafter. The initial content of each plant was estimated from its transpiration during the treatment period: this provided a basis for calculating the loss during the period after the end of treatment. When the griseofulvin was determined chemically, the amount taken up was determined from the loss of griseofulvin from the treating solution and the amount in the plants was determined a known period after treatment had ceased. The bioassay estimations allowed sufficient replication to determine statistically the general form of the decay curve but the errors in the method were reflected in the estimation of its constants. The chemical methods were too laborious to allow sufficient replication to determine the form of the curve but permitted a more accurate determination of its constants.

The results of two decay experiments in which the griseofulvin contents were determined by bioassay are illustrated in Fig. 2. The logarithm of the percentage of the original griseofulvin remaining after t days has been plotted against the time in days. The data from both experiments could be fitted by the same straight line

$$\log_{10}\left(\frac{100w}{W}\right) = 1.97 - 0.05t \quad (1)$$

where W is the initial and w is the final griseofulvin content and t is the period of breakdown in days. The constant 1.97 is a statistical approximation to $\log_{10} 100$ and the decay equation can be presented in the more general form

$$w = W.e^{-kt} \quad (2)$$

where k is the decay constant. The value of the constant determined from the data in Fig. 2 is 0.12. A third bioassay experiment yielded a value of 0.17 for

50 *Crowdy, Grove, Hemming, Robinson—The Translocation of*
the decay constant: the data, however, were fitted by a curve of the same type.
The amount of decay, dw , in a time, dt , is given by the equation

$$dw = -kw \cdot dt. \quad (3)$$

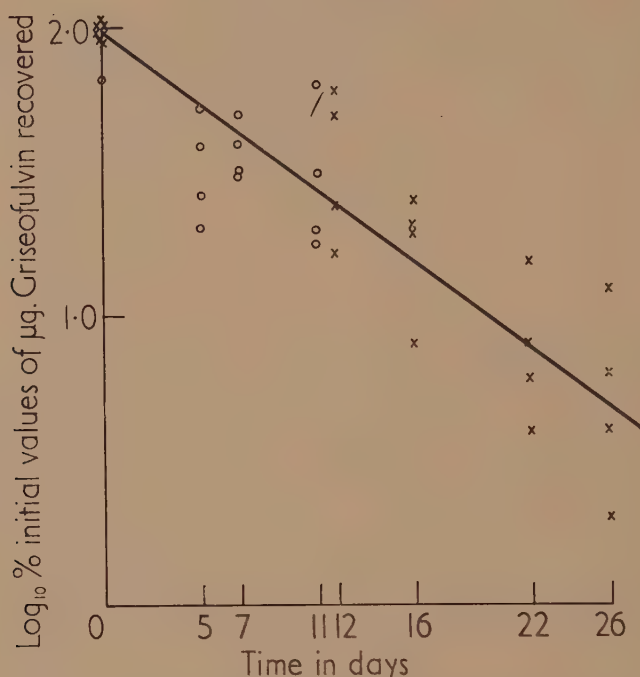


FIG. 2. Decay of griseofulvin in broad bean: \log_{10} percentage of original content present after a period of time.

$$\begin{aligned} \text{Experiment A} - \circ & \left\{ \log_{10} \left(\frac{100w}{W} \right) = 1.97 - 0.05t. \right. \\ \text{Experiment B} - \times & \end{aligned}$$

Accumulation in the tissues

The rate at which griseofulvin accumulates in the tissues will depend on the rate of uptake and the rate of decay. Since the former is probably linear and the latter is exponential the rate of accumulation cannot, in fact, be linear and the constant rates of accumulation recorded above represent statistical approximations to the true picture. However, for short time periods e^{-kt} (equation 2) will tend to unity and w will tend towards W : in this case the linear relation between observed accumulation and time will reflect a real linear relation between uptake and time. Owing to the errors of the bioassay technique it is probable that a considerable deviation of e^{-kt} from unity would escape detection. This observed linear relationship between uptake and time must also be considered in the context of the experimental conditions, which imposed a constant rate of transpiration over the period of the experiment. It has already been noted that the uptake of griseofulvin is related to transpiration: in these circumstances the constant rate of uptake with time will occur only in conditions of constant

transpiration and the accumulation equations derived below are only applicable in these conditions.

In these circumstances, the total uptake of griseofulvin, W , after a time, t , can be expressed by the equation

$$W = a + bt \quad (4)$$

where a is the initial uptake by the roots and b is the rate of uptake with time. The amount of griseofulvin, dw , taken in over a period of time, dt , is given by the equation

$$dw = b \cdot dt. \quad (5)$$

This relation has already been assumed in calculating the data shown in Table V.

The rate of accumulation when the supply of griseofulvin is continuous, dw/dt , is the sum of the rate of uptake and the rate of decay

$$\frac{dw}{dt} = b - kw. \quad (6)$$

The weight of griseofulvin in the plant, w , at a given time can be calculated from the integral of equation (6) assuming that the content at zero time is a

$$w = \left(a - \frac{b}{k}\right)e^{-kt} + \frac{b}{k}. \quad (7)$$

Estimates of the decay constant, k , were also derived from the experiments in which the weight of griseofulvin removed from the treating solution, W , and the amount in the plants at the end of the experiments, w , were determined chemically. In these experiments, an initial period of treatment, T days, with griseofulvin solution was followed by a decay period, t days, during which the plants were held in water. The weight of griseofulvin, w_d , accumulated by the end of the treatment period provided the capital for the decay period. From equation (7)

$$w_d = \left(a - \frac{b}{k}\right)e^{-kT} + \frac{b}{k}$$

and

$$w = w_d \cdot e^{-kt} = \left[\left(a - \frac{b}{k}\right)e^{-kT} + \frac{b}{k}\right]e^{-kt}. \quad (8)$$

A value for k can be obtained by solving equation (8); the constants required for this solution are shown in Table VI.

In all the experiments, the concentration used for treatment was 25 $\mu\text{g./ml.}$ and the initial uptake has been estimated by assuming that the roots attain the concentration of the treating solution during the initial period. The rate of uptake, b , was calculated from equation (4). The estimates of k agree fairly well among themselves and also agree well with one of the bioassay trials. It seems reasonable to assume that the decay constant was approximately the same in these trials and has a value of 0.18 which is the mean of the two long term trials assayed chemically. With this value for the constant, the half life of griseofulvin in the plant is about 4 days.

TABLE VI

Calculation of Decay Constant in Broad Bean Plants from Experiments in which Griseofulvin was Estimated Chemically

(Experiment numbers correspond to those listed in Table V)

Time in days			Griseofulvin				
Expt. no.	Treat.	Decay	Total uptake mg.	Final content mg.	Initial uptake mg.	Uptake rate mg./day	Decay const.
	<i>T</i>	<i>t</i>	<i>W</i>	<i>w</i>	<i>a</i>	<i>b</i>	<i>k</i>
2	3	0	41	31	9	11	0.17
3	3.5	6.5	26	5	7.0	5.4	0.19
4	2	14	51	4	7.6	21.7	0.17

* Experiment described by Crowdy *et al.* (1955).

The possible effect of griseofulvin leaching from the roots of bean plants standing in water has been ignored in the foregoing observations on decay. This leaching effect might, however, exaggerate the rate of decay. In practice, it is difficult to draw a clear distinction between thorough washing and leaching. In experiment 3 of Table VI, an attempt was made to estimate leaching losses by determining the griseofulvin (*a*) in the water used for the last washing before transferring the plants for growing on water (2 mg.), (*b*) in the water in which the plants had been growing for the first 2.5 days after the end of treatment (8 mg.) and (*c*) in the water in which the plants had been growing from 2.5 days to 4 days (0 mg.). The recoveries of griseofulvin at these stages indicates that an appreciable weight was returned to the solution surrounding the roots during the first 2.5 days but none thereafter. The 8 mg. returned to the solution was not included as part of the 'total uptake' recorded in Table VI which was used in the estimate of the decay constant: even with this omission this experiment gave the highest value of the decay constant in the series. This suggests that the loss was abnormal in this case and may have been due to ineffective washing rather than leaching. The value of the decay constant was identical for experiments 2 and 4 in Table VI though in the first there was no period of growth on water when leaching would have taken place and in the second no allowance was made for leaching. Further this value agreed well with one of the bioassay trials. It will be shown below (Table VII) that griseofulvin moves predominantly from the roots to the shoots and this may account for the minor importance of leaching.

It is of interest to compare the accumulation of griseofulvin calculated from equation (7) with that actually recorded. This comparison is shown in Fig. 1: the straight lines describe the observed accumulation of griseofulvin with time by Sutton's Dwarf broad beans from solutions of various concentrations while the points show the values of *w* obtained by solving equation (7) for the

appropriate time intervals. The constants required for this solution were obtained as follows:

- a* the initial content was obtained by extrapolating the experimental lines to zero time;
- b* a value of $17.7 \mu\text{g./ml.}$ water taken up from a solution of $25 \mu\text{g./ml.}$ was obtained from the mean of the observations recorded in Table V. In this experiment, the rate of transpiration was 0.72 ml./day giving a rate of uptake of $12.7 \mu\text{g./day}$. For the lower concentrations of treating solutions, the rate of uptake was taken to be proportional to the concentration of the treating solution.
- k* 0.18 was taken as the value of the decay constant.

Fig. 1 illustrates that in this experiment there was good agreement between the calculated and observed results and that for the experimental conditions and times used constant accumulation rates provide a reliable picture of accumulation. The agreement also provided additional evidence of the validity of the assumptions made in solving equation (7).

Distribution of griseofulvin within the plant

Evidence has already been produced to show that the rate of griseofulvin accumulation is determined by transpiration and the concentration of the treating solution. These relationships are further illustrated in Figs. 3 and 4 which include data from experiments with both broad bean and tomato. For the sake of clarity the actual observations on tomato have been omitted, only the regression line has been included. A straight line adequately described the relationship between griseofulvin accumulation and transpiration in all three experiments, and tomatoes behaved in much the same way as beans. Fig. 4 illustrates a linear relationship between the accumulation after a definite time and the concentration of the treating solution. The distribution of griseofulvin within root-treated broad beans and tomatoes is illustrated in Figs. 5 and 6. The plants were sampled over a period of 138 hours and at each sample the upper and lower leaves, stems, and roots were extracted and assayed separately. The experiments were carried out at different times and quantitative comparisons between them are not justified. There were, however, qualitative differences between the behaviour of the two plants. In both beans and tomatoes, the concentration of griseofulvin was initially higher in the stems than in the leaves and remained more or less constant throughout the trial. In beans, the initial concentration in the roots was higher than that in the stems but only increased slowly; the main accumulation was in the leaves, especially the lower leaves. In tomato, on the other hand, the main accumulation was in the roots: there was marked accumulation in the leaves but there was little difference between the behaviour of the upper and lower leaves.

If, after an initial treatment period, the supply of griseofulvin was removed and the plants were allowed to grow for a period with their roots in water, rather a different picture was presented as is illustrated in Tables VII

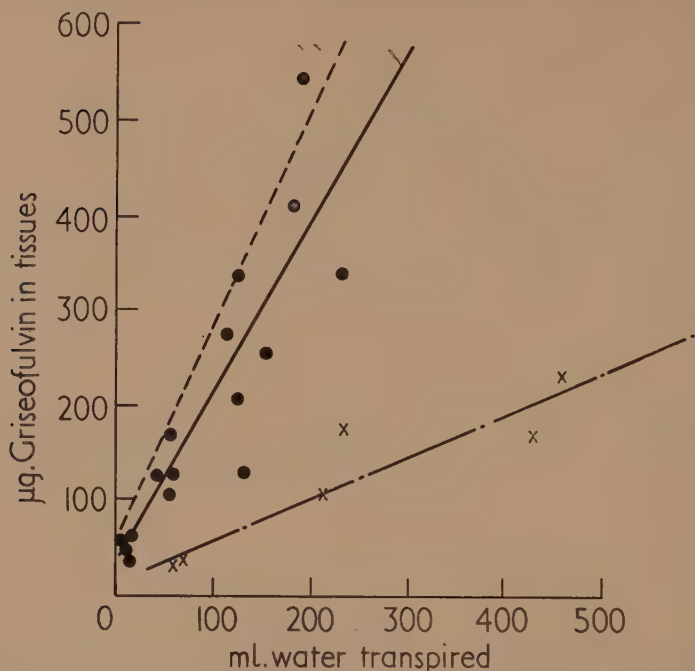


FIG. 3. Relationship between water uptake and griseofulvin recovery in beans and tomato. Treatments: Bean 3 $\mu\text{g}/\text{ml}$. $x \cdots x$ $W = 0.42x + 19$; Bean 6 $\mu\text{g}/\text{ml}$. $\bullet \cdots \bullet$ $W = 1.75x + 34$; Tomato 6 $\mu\text{g}/\text{ml}$. $-----$ $W = 2.1x + 72$

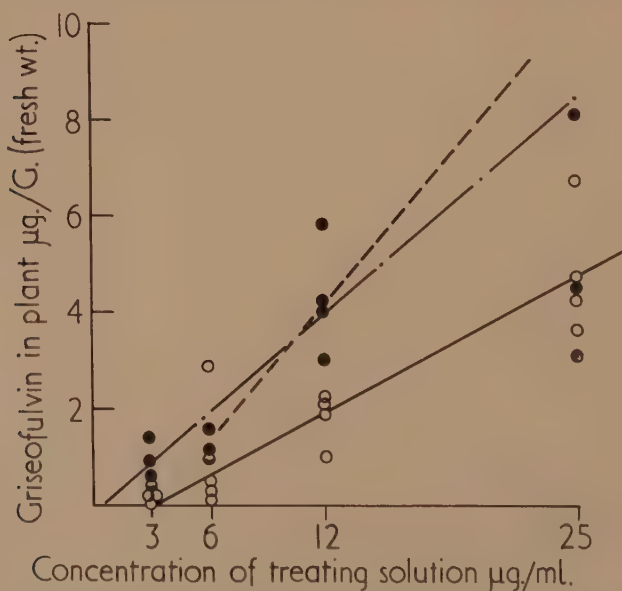


FIG. 4. Effect of concentration in solution on accumulation of griseofulvin by broad bean and tomato. $\bullet \cdots \bullet$ Broad Bean 4 days ($Y = 0.34x - 0.16$); $\circ \cdots \circ$ Broad Bean 8 days ($Y = 0.21x - 0.65$); $-----$ Tomato 3 days ($Y = 0.47x - 1.47$)

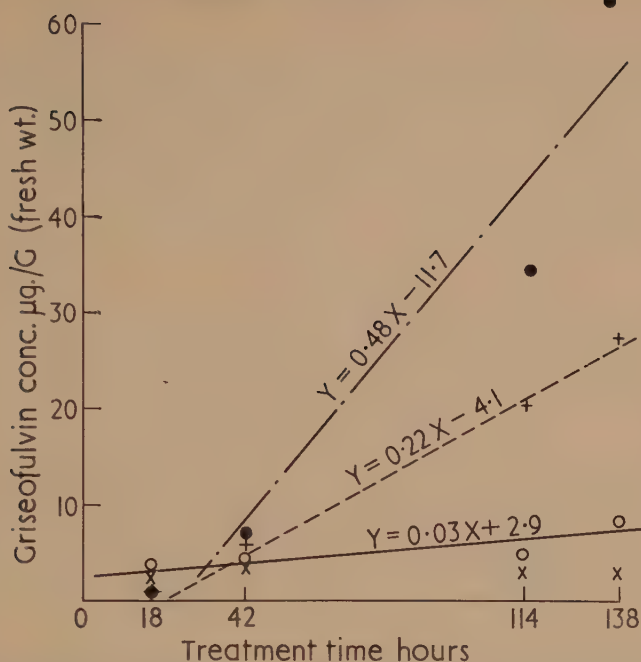


FIG. 5. Concentration of griseofulvin in $\mu\text{g./G}$ (fresh wt.) in bean plants treated with griseofulvin $6 \mu\text{g./ml.}$ Root + - - - +; Stem x x; Upper Leaves ●—●; Lower Leaves ○—○

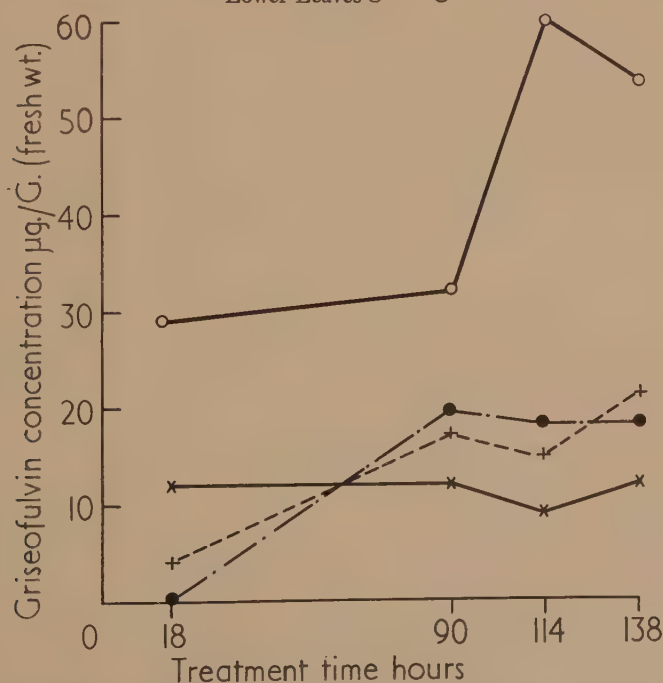


FIG. 6. Concentration of griseofulvin in $\mu\text{g./G}$ (fresh wt.) in tomato plants treated with $6 \mu\text{g./ml.}$ of griseofulvin. Root ○—○; Stem x—x; Upper Leaves + - - - +; Lower Leaves ●—●

and VIII. In this experiment, the plants were held in a solution of 25 $\mu\text{g./ml.}$ griseofulvin for 6 hours, the roots were then washed and the plants were transferred to water and sampled at intervals. The plants in each sample were divided into 7 sections: the leaves and stems at 3 different levels and the roots. The leaves and stems of the top section are referred to as 'a'. The general trend of the results is shown in Table VII in which the total griseofulvin assayed in the stems, leaves, and roots at each sampling time are shown.

TABLE VII

Weight of Griseofulvin Assayed in the Leaves, Stems, and Roots of Broad Bean Plants at Various Times after the Cessation of Treatment

Time after treatment hours	Weight griseofulvin $\mu\text{g.}$		
	Leaves	Stems	Roots
0	3	28	279
21	31	18	39
40	44	7	0
64	27	0	0
128	56	1	0

This table shows that there was an increase of griseofulvin in the leaves which was matched by a decrease of griseofulvin in the roots and stems. Total weight figures do not provide a satisfactory basis for comparing distribution since they are subject to variation in initial uptake and loss by decay. For these reasons, the distribution of griseofulvin at each sampling time, calculated as a percentage of the total griseofulvin assayed in the whole plant, is shown in Table VIII. For statistical analysis these percentages were transformed to angles and these transformed percentages are shown in Table IX since the significant differences between them can be appreciated more readily.

TABLE VIII

Distribution of Griseofulvin in Broad Bean Plants at Different Times after the End of Treatment

Time after treatment, hours	Percentage griseofulvin						Roots
	Leaves			Stems			
	a	b	c	a	b	c	
0	0.7	0	0.3	0.3	1.6	6	91
21	15	10	10	6	10	4	44
40	48	20	20	4	5	4	0
64	21	58	21	0	0	0	0
128	15	35	48	2	0	0	0

TABLE IX

Distribution of Griseofulvin in Broad Bean Plants at Different Times after End of Treatment. Percentages of Total Griseofulvin Assayed Transformed to Angles

	Transformed percentage griseofulvin						
	Leaves			Stems			Roots
Time after treatment, hours	a	b	c	a	b	c	
0	4	0	2	2	7	14	73
21	23	18	18	14	18	11	42
40	44	25	25	8	12	8	0
64	27	45	27	0	0	0	0
128	23	34	44	6	0	0	0

Least significant difference is 16 ($P=0.05$)
Least significant difference is 22 ($P=0.01$)

After the initial treatment period, most of the griseofulvin was in the roots, though there was an appreciable proportion in the base of the stem. As the time after treatment increased, the griseofulvin was removed first from the roots and then from the stems till it finally accumulated in the leaves. In the earliest sample, there was a suggestion of a higher concentration in the base of the stem but this was not significant. There was also a tendency for the highest proportion of griseofulvin to be in the top leaves at 40 hours, in the centre leaves at 64 hours, and in the bottom leaves at 128 hours. A parallel trend was shown by the concentration, in $\mu\text{g./g.}$, in the leaves at these times but in this case the differences were not significant. The data do not indicate the significance of this trend. It is clear, however, that the griseofulvin did not move from the lower leaves towards the growing point when the reserve in the roots had been exhausted. If the roots were cut off and cut shoots were immersed in the solution of griseofulvin, the behaviour was essentially the same as that of rooted plants. When sampled immediately after treatment, 74 per cent. of the griseofulvin was to be found in the two basal nodes of the stem while there was 20 per cent. in the leaves; after standing in water for 24 hours, the basal nodes of the stem contained only 13 per cent. of the antibiotic, a quantity comparable with the remainder of the stem, while the leaves contained 76 per cent. of the total found. This proportion was substantially unaltered at the end of a further 24 hours.

A similar experiment in which tomato plants were transferred to water after an initial treatment of 18 hours in 25 $\mu\text{g./ml.}$ of griseofulvin is illustrated in Tables X and XI.

In another similar experiment with tomatoes in which the plants received an initial treatment for 6.5 hours and were then grown on water and sampled daily for 7 days the percentage distribution did not change significantly after the first 24 hours after treatment and the distribution at the end of 7 days was: leaves, 24 per cent.; stems, 40 per cent.; roots, 36 per cent. These may be

TABLE X

Distribution of Griseofulvin in Tomato Plants at Different Times after the End of Treatment

Time after treatment hours	Percentage griseofulvin				Roots
	Leaves		Stems		
	a	b	a	b	
0	14	14	17	23	33
24	47	19	8	13	13
48	48	25	2	10	16

TABLE XI

Distribution of Griseofulvin in Tomato Plants at Different Times after the End of Treatment. Percentage Distribution of Total Griseofulvin Assayed Transformed to Angles

Time after treatment hours	Transformed percentage of griseofulvin				
	Leaves		Stems		Roots
	a	b	a	b	
0	21	21	23	29	35
24	44	26	16	21	21
48	44	30	6	18	23

Least significant difference is 12 ($P = 0.05$)

compared with the corresponding figures after 48 hours for the experiment illustrated in Table X: leaves, 72 per cent.; stems, 12 per cent.; roots, 16 per cent. The data suggest two differences between the behaviour of the antibiotic in tomatoes and beans; in the latter all the griseofulvin moved out from the roots and later the stems and tended to accumulate in the leaves, while in the former a considerable proportion remained in the stems and roots, though there was a tendency for some to move into the leaves. There appeared also to be a tendency for accumulation in the upper leaves of tomato. The second trial with tomato also indicated that with smaller doses the proportion of griseofulvin retained in the roots is higher,

The effect of respiratory inhibitors on the uptake of griseofulvin by the broad bean

Stokes (1954) reported that the use of certain respiratory inhibitors in the culture solution could prevent the appearance of griseofulvin in the guttation drops of wheat seedlings. The effect of two of these inhibitors, sodium azide and 2:4-dinitrophenol, on the uptake of griseofulvin by the bean has been investigated. It was found that each of these inhibitors could be added to the culture solution at a concentration of 10^{-5} without affecting either the water uptake or the fresh weight of the treated plants over a period of 93 hours and this concentration was used in treatment. The plants were treated initially for

18 hours in solutions of these inhibitors and were then transferred to a solution containing the inhibitor and $6\mu\text{g.}/\text{ml.}$ of griseofulvin; the control plants were treated in water for the 18-hour period and then transferred to a solution of griseofulvin without the inhibitor. Treatment was continued for 75 hours and over this period the total water transpired was a linear function of time and the transpiration rate was the same for all treatments.

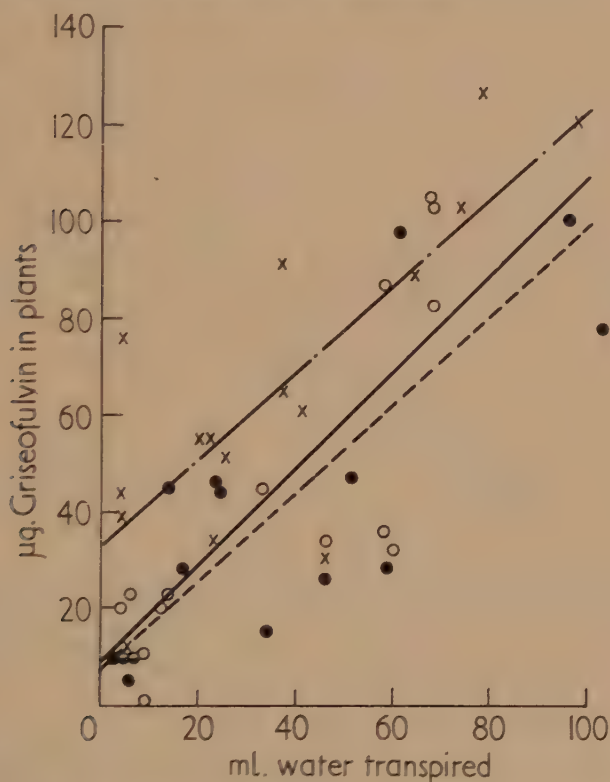


FIG. 7. Relationship between total griseofulvin found in bean plants treated with water, Sodium Azide and Dinitrophenol, and the water transpired. Sodium Azide \circ ----- $Y = 0.93x + 7$; 2:4-Dinitrophenol \bullet — $Y = 1.01x + 9$; Water \times — · — $Y = 0.91x + 33$.

Fig. 7, in which the total weight of griseofulvin is plotted against the total water transpired, illustrates the general effect of the respiratory inhibitors. There was a linear relationship between the total water transpired and the total griseofulvin assayed in the plants. There was no significant difference between the rate of uptake of griseofulvin by the plants, as is shown by the slope of these lines, but the difference between the position of the lines is significant. Within the limits of error the lines representing the uptake of the plants treated with sodium azide and dinitrophenol pass through the origin while there is a significant initial content of griseofulvin in the plants which were not

treated with inhibitors. This indicates that the respiratory inhibitors markedly reduced or extinguished the initial rapid uptake of griseofulvin by the roots but did not affect the rate of entry with the transpiration stream.

The effect of respiratory inhibitors on movement into the shoots is illustrated in Fig. 8. The total griseofulvin in the shoots has been plotted against the total water transpired: the relationship again is linear. Within the limits of

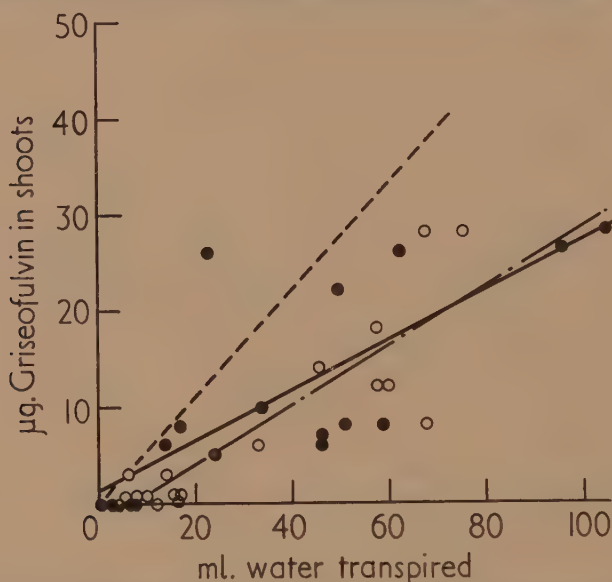


FIG. 8. Relationship between the griseofulvin found in the shoots of bean plants treated with water, sodium azide and 2:4-Dinitrophenol, and the water transpired. Sodium Azide $\bigcirc - \cdot - \bigcirc$ $Y = 0.30x - 1.7$; 2:4-Dinitrophenol $\bullet - \bullet$ $Y = 0.26x + 1.5$; Water $- - - -$ $Y = 0.55x + 0.3$.

error all three lines pass through the origin but the rate of transfer to the shoots was significantly greater for the water treated plants than for those treated with sodium azide and dinitrophenol. In all treatments, the rate at which the griseofulvin was carried to the shoots in the transpiration stream was significantly less than the rate at which it was accumulated by the whole plant; the lower rate of transport to the shoots in the water-treated plants must be attributed to absorption of griseofulvin from the transpiration stream by the roots. Since the general picture presented in Fig. 7 indicates that these respiratory inhibitors did not affect the rate of movement with water, the further reduction in the rate of transport to the shoots in the inhibitor-treated plants probably reflects a greater absorptive capacity in the roots of these plants, which is the result of the deficit following the inhibition of the initial uptake; however, the possibility of a direct effect of the inhibitors is not excluded.

Griseofulvin in expressed sap

Crowdy and Pramer (1955) have already noted that griseofulvin occurred in the expressed sap of beans and tomatoes and that the concentration in the sap

was considerably lower than the concentration in the tissue residues left after pressing. This is further illustrated in Tables XII and XIII. Table XII illustrates the effect of time on the concentration of griseofulvin in expressed sap and in the residues of leaves of Express broad beans and tomatoes treated with a solution of 6 $\mu\text{g./ml.}$ of the antibiotic.

TABLE XII

Concentration of Griseofulvin in Expressed Sap ($\mu\text{g./ml.}$) and in Residues ($\mu\text{g./g. fresh wt.}$) after Various Treatment Periods on a Solution of 6 $\mu\text{g./ml.}$

Time hours	Griseofulvin concentration							
	Express beans				Tomato			
	Upper leaves		Lower leaves		Upper leaves		Lower leaves	
	Sap	Residue	Sap	Residue	Sap	Residue	Sap	Residue
18	0	0.6	0.05	1.5	0	2.5	0	4
42	0.05	10.0	0.25	14.7	—	—	—	—
90	—	—	—	—	0.05	27.5	0.1	43.5
114	0.5	41.3	0.6	66.5	0.15	18.0	0.1	34.0
138	0.7	62.3	0.9	140.8	0.45	32.5	0.25	38.5

Correlation coefficient (r)

relating conc. in sap

to conc. in residue 0.79 ($P < 0.001$)

0.17 (not significant)

Table XIII illustrates an experiment in which Sutton's Dwarf broad bean plants were treated for 4 days and tomatoes were treated for 3 days with a range of concentrations of griseofulvin.

TABLE XIII

Concentration of Griseofulvin in Expressed Sap ($\mu\text{g./ml.}$) and in Residues ($\mu\text{g./g. fresh wt.}$) after Treatment on Solutions of Different Concentrations

Treat- ment concen- tration $\mu\text{g./ml}$	Griseofulvin concentration							
	Dwarf beans				Tomato			
	Upper leaves		Lower leaves		Upper leaves		Lower leaves	
	Sap	Residue	Sap	Residue	Sap	Residue	Sap	Residue
25	2.4	72.5	4.0	123.5	11.0	93.5	9.4	99.0
12	1.6	46.5	2.0	43.0	2.0	73.8	5.5	39.3
6	0.2	22.5	0.2	28.0	1.6	22.5	1.6	20.8
3	0	12.0	0.2	12.0	0.7	21.0	1.1	16.0

Correlation coefficient (r)

relating conc. in sap

to conc. in residue 0.81 ($P < 0.001$)

0.56 ($0.02 > P > 0.01$)

The data from these tables show that with beans the concentration of griseofulvin in the sap was highly correlated with the concentration in the residues. This correlation was also significant in two other experiments not presented here ($r = 0.76$ and $r = 0.58$); in a third there was no correlation ($r = 0.04$). For tomatoes, the correlation was significant in only one of the experiments presented: of two experiments with tomatoes not presented here, only one showed a significant correlation ($r = 0.74$). When the data relating the concentration of griseofulvin in the sap to the concentration in the residue were combined for all the experiments with beans the correlation was low but significant ($r = 0.37$, for 92 pairs of observations). A similar comparison for tomato showed no correlation ($r = 0.18$ for 76 pairs of observations).

If the relative concentrations of griseofulvin in the residues and in the sap were determined by a simple physical effect such as partition between the water and the lipoids of the cell a high correlation would have been expected between the concentration in these two fractions in the combined data of all the experiments relating to a particular variety. The low correlation recorded with beans and the absence of correlation with tomato indicated that this relationship was profoundly influenced by undefined differences between experiments suggesting that the balance is also determined by other factors. No evidence of covalent bond formation has been found (Crowdy *et al.*, 1955) and the present data throw no further light on the effect.

DISCUSSION

The data presented above give a general picture of the uptake and accumulation of griseofulvin in broad bean plants. Two processes appear to be involved in entry into the roots. The first might be described as a passive movement of the compound in the transpiration stream through the roots and into the shoots. The second process, however, seems more complicated. Within a short time, probably less than 30 minutes at the temperature used, there was a rapid equilibration between the concentration of griseofulvin in the roots and that in the surrounding solution; this was followed by a steady increase in the concentration of griseofulvin in the roots to values considerably higher than that in the solution which surrounded them. This form of uptake occurred in detached roots and was independent of transpiration. Sodium azide and 2:4-dinitrophenol, at concentrations which did not interfere with transpiration, did not interfere with the first process, but they markedly reduced or inhibited the initial rapid uptake. This inhibition, however, did not appear to prevent the roots of inhibitor treated plants from accumulating griseofulvin from the transpiration stream since there was a significant reduction in the rate of flow of griseofulvin to the shoots of these plants. While the effect of inhibitors may indicate the operation of an active process, there is no evidence of accumulation of griseofulvin against a concentration gradient. The high concentrations recorded in the roots are probably attributable to the greater solubility of the

compound in lipid than in aqueous media. There is no evidence of high concentrations in the expressed sap. The effect of inhibitors may have been indirect, possibly altering the permeability of the cells to griseofulvin; it should be noted, however, that this indirect effect did not influence the rate of entry in the transpiration stream.

It is probably unwise to press comparisons between the uptake of ions and the uptake of neutral substances such as griseofulvin but the general course of uptake described above is similar in some respects to the picture of the uptake of calcium and chloride ions by pea plants described by Hylmö (1953). Hylmö found that ion uptake by the whole plant was related to the water transpired and to the concentration of the treating medium. There was an initial uptake both by whole plants and by detached roots which was inhibited by low temperatures, but low temperature did not affect the movement associated with transpiration.

Movement of griseofulvin from roots to shoots seems to be dependent on transpiration and the compound tends to accumulate in the leaves. In broad beans the accumulation is most rapid in the lower leaves and, if the source of griseofulvin is removed, all or most of the griseofulvin is transferred from the roots to the leaves. In tomato, the greatest accumulation is in the roots though there is some accumulation in the leaves: if the source of griseofulvin is removed, there is some transfer from the roots to the shoots, but a high proportion is retained in the roots.

A logarithmic decay of griseofulvin has been demonstrated in bean tissue and in the conditions of these experiments decay has been relatively constant. Though the form of the decay curve is probably characteristic, more varied experimental conditions might have revealed more variation in its constants.

It is also possible to make some general comparisons between the movement of griseofulvin in broad bean and tomato and the movement of chloramphenicol and streptomycin in these two plants and in cucumber (Pramer, 1953, 1954). There is a general similarity between the behaviour of griseofulvin and chloramphenicol in the shoots of broad bean and tomato since the latter tended to accumulate in the shoots of these plants. In contrast, there was little accumulation of chloramphenicol in the shoots of cucumber. Streptomycin, on the other hand, accumulated freely in the shoots of cucumber and tomato but only appeared as traces at the base of broad bean shoots after prolonged treatment. These comparisons indicate the range of variation which may be expected when considering the translocation of antibiotics in higher plants.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. D. G. Christie for the mathematical interpretation of the decay data, and Miss A. V. Petrie, Mr. D. Gardner, and Mr. B. Langford for assistance in the experimental work involved.

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Studies of Growth and Development in the Genus *Fragaria*

VI. THE EFFECT OF PHOTOPERIOD AND TEMPERATURE ON LEAF SIZE

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Received 25 July 1955

SUMMARY

It has been demonstrated that photoperiod plays the dominant role in bringing about the reduction in leaf size and in the number of cells in the shortening days of autumn. Strawberry plants in various physiological conditions all respond similarly by prolonging the period of cell division in expanding leaves in response to long-day treatment, and by curtailing the period of cell division in short days. At the lowest temperatures within the normal range for active growth there is a reduction in leaf size and in cell size, and a less marked reduction in cell number. The effect of artificial illumination is shown to depend on the duration of the daily light period, and not on the total daily dose of light. Light intensity has no effect on leaf growth over the ranges used, either from fluorescent lamps or from high pressure mercury vapour lamps. The relation between the master reaction determining the cessation of cell division, and that determining the cessation of vacuolation is discussed.

INTRODUCTION

AN analysis of the parts played by cell division and cell enlargement in the seasonal variation in leaf size of the cultivated strawberry (Arney, 1953, 1954) led to the conclusion that leaf size was mainly controlled by duration of the cell-division phase in the young leaf, and that differences in cell size played only a minor role, being responsible for less than one-quarter of the total variation in leaf size. The results suggested that the master reactions governing the termination of cell division and of cell vacuolation were probably not consecutive, since there is often a negative correlation between the duration of the cell-division phase and the final size of the cells. A series of experiments on the effect of artificial illumination and heat on the growth of the strawberry plant has afforded the opportunity to obtain data on the effect of these factors on leaf growth, thus supplementing some experiments directly aimed at showing the part played by photoperiod in the seasonal variation in leaf size. Analysis of these results might also be expected to yield further information on the relationship between the master reactions controlling cell division and vacuolation.

EXPERIMENTAL DETAILS

Virus-free Royal Sovereign runners less than one year old have been used throughout; most of these plants were obtained from commercial nurseries under the Ministry of Agriculture certification scheme, but a few healthy offspring from virus-free plants bought in a previous year have also been used. The runners were planted in tomato baskets 15 × 9 in., which could be placed close together, yet allowing free circulation of air between the slightly tapering sides of the baskets. All plants were given a weekly dose of 0.2 g. of KNO_3 ; 0.08 g. of KH_2PO_4 ; 0.08 g. of K_2HPO_4 mixed in solution. This was supplemented with Hoaglands A-Z solution at the normal strength recommended for solution cultures. The leaflets were uniformly dark green and showed no visible mineral deficiency symptoms.

The apical leaflet was used in all cases for the cell counts and leaflet area determinations; the cell counts were made on the upper epidermis after clearing in lactic acid (for details, see Arney, 1954). Leaflets were not detached from the plant until they were beginning to turn yellow, or until the plant was dissected at the end of the experiment. This avoids any possible effect of partial defoliation.

Additional illumination was obtained from mercury vapour lamps, either fluorescent tubes or high-pressure horticultural illuminators with aluminium reflectors. Ekco daylight 80 watt fluorescent tubes were used without reflectors; the strips were supported at a height of 9 in. above the soil in the baskets by means of wire holders at each end, the control gear being fixed to the switch-board. The fluorescent tubes were arranged parallel to each other at a distance of 18 in., to cover the whole area occupied by the plants. Although this arrangement involved just over 50 per cent. loss of light through upward radiation, the absence of reflectors cut down the obstruction of daylight to very small proportions. This method gave an additional light intensity of 1,600 lux (excluding daylight) at the leaf surface. In another greenhouse four 500-watt high-pressure mercury vapour lamps were used at a height of 5 ft. above the leaves of the plants; two 250-watt tungsten filament lamps were also placed centrally to provide red light. Altogether these lamps produced a light intensity, at plant level, of approximately 2,000 lux (exclusive of daylight). The daylight intensity inside the greenhouse varied widely with weather conditions, but amounted to just over 1,000 lux under average winter conditions, at noon, with an overcast sky. The total light intensity was thus maintained at between 2,000 and 3,000 lux throughout most of the daylight period, and never fell below 2,000 lux, even after dusk. The lights were left on throughout the whole of the light period, from before dawn till after dusk, except during periods of direct sunlight.

In the autumn and winter of 1953 some plants were illuminated out of doors by mounting two 1,000-watt floodlights at a height of 3 ft. above ground level on a wooden platform which sloped steeply forward so that the light was thrown down on to the plants arranged in an area approximately

3 yds. square, at a distance of from 4 to 13 ft. from the floodlights. This produced a light intensity of 2,500 lux at the edge nearest to the lamps, and 1,250 lux at the farthest edge. The lamps were switched on before sunset, and switched off again at a time which made the total duration of the light period exactly 18 hours; as the time of sunrise became later, the time at which the lights were switched off was also made later. It was found that the minimum night temperature at plant level in this illuminated area was often as much as 1° C. above the minimum night temperature at a distance from the lamps. In the autumn of 1954 some H.P.M.V. lamps were used to illuminate plants out of doors, growing in a border facing eastwards, and backed by a wall on the west side. The control (unilluminated) plants were grown in the middle stretch of the border, separated by a distance of 4 yds. from the two illuminated beds at each end of the border; these control plants behaved in the same way as comparable plants grown in another unilluminated bed some miles away. One end of the bed was illuminated by two H.P.M.V. lamps from 1.30 to 2.15 every night from 14 August onwards, while the other end of the bed was illuminated by two similar H.P.M.V. lamps from sunset onwards, the time of switching off being progressively later to maintain an 18-hour photoperiod. No difference in minimum night temperature could be detected between the illuminated and control beds. The supplemental light intensity after dark was 2,000 lux over most of the experimental area, but only 1,000 lux at the corners.

A much lower intensity of red light than of blue light is required to initiate photoperiodic reactions in the strawberry (Roodenburg, 1954). But previous experience shows that the light intensity of late autumn and winter is insufficient to sustain the vigorous growth of Royal Sovereign plants which would otherwise be expected in a heated greenhouse, and for cheapness and efficiency mercury vapour lamps were used to obtain the high light intensities required for vigorous growth in the greenhouse; similar illumination was therefore used outside, for the sake of comparability, although a much lower intensity of red light would undoubtedly have been as effective in producing the photoperiodic reactions.

ANALYSIS OF THE RESULTS

Wherever possible the leaflets were sampled in such a way that the data could be subjected to an analysis of variance appropriate for a multifactorial experiment, in which photoperiodic treatment, temperature, sampling occasion, and the individual plant from which the leaf was taken were the 'factors', at two or more 'levels'. Previous analysis on these lines had shown that individual plants sometimes had characteristic differences in leaf size over a considerable period of time (Arney, 1954, p. 351), but in only a few plants were these differences significantly greater than the random differences (including measurement errors) between successive leaves on the same plant. In the data considered in this paper there is only one experiment in which these systematic differences between individual plants reach statistical significance. It can be

concluded that there is something to be gained in reliability by including leaflets from as many different plants as possible in each sample, rather than replicating with two leaves from one plant, or two cell counts on a single leaf.

The sets of plants for the various treatments in any experiment were selected at random from the same batch, and were all grown in identical conditions for an initial period during which leaf samples were obtained at fortnightly or monthly intervals; the various batches were then removed to their respective treatments and leaf sampling continued. Thus the differences due to treatment can only develop towards the end of the period of observation, and the statistical significance of the effect of the various treatments should be estimated from the significance of the treatment–occasion interaction, and not from the treatment variance. The analysis of variance for an experiment with T treatments, and leaf samples taken on M different occasions from the N plants in each treatment would be as follows:

Between plants:	Degrees of Freedom
Between treatments	$T-1$
Within treatments	$T(N-1)$
Within plants:	
Between occasions	$M-1$
Occasion–treatment interaction	$(M-1) \times (T-1)$
Residual	$MNT-1-[T-1+M-1+T(N-1)+$ $\qquad\qquad\qquad+(M-1)(T-1)]$
TOTAL	$MNT-1$

The significance of the treatment–occasion interaction is tested against the ‘within plants’ residual.

THE EFFECT OF PHOTOPERIOD ON LEAF SIZE

The effect of photoperiod has been observed during the winter by applying supplementary illumination to plants which had previously been growing in the short natural daylengths. No attempt was made to produce short-day conditions during the summer months, because it would have been impossible to keep the plants sufficiently cool under light-proof covers with the facilities available. In all cases a change to long days has resulted in an increased size of the leaves expanding immediately after the change, and of all leaves developed subsequently, in the long days. Treatments *B*, *C*, and *F*, of Table I delayed dormancy by applying long days from August onwards; treatment *B* has given similar results, under H.P.M.V. lamps during the 1954–5 season (Fig. 1). Since strawberry plants become inactive during the winter months, and long-day treatment forces them into growth again, it is important to distinguish between the direct effect of long days specifically on leaf size, and any possible indirect effect through the forcing action of long days on growth as a whole. In treatments *C* and *F* in Table I, which involved growing the plants in a heated greenhouse under long days, growth activity was kept at a uniform level from August onwards, and there can be no possibility of any dormancy-breaking effect of the long-day treatment. In all these treatments the contrast

between the constant level of leaf size in the long days, and the marked decline in leaf size under natural (short) daylengths, is statistically significant at the 1 per cent. level.

In treatments *D* and *E* of Table I the trend induced by the seasonal shortening of the daylength was subsequently reversed by application of long days

TABLE I

The Effect of Photoperiod on Leaflet Size, Cell Division, and Vacuolation

- A.* Growing under normal autumn conditions in beds, or in baskets outside.
B. Grown outside in baskets and pots, with supplemental illumination to give 18 hrs. light daily from 14 August onwards.
C. Transferred from treatment *B* to a heated greenhouse under 18 hrs. light daily on 5 October.
D. Grown outside in baskets and transferred to the heated greenhouse with 18 hrs. light daily on 9 October (see also Table III B).
E. As *D* but transferred to the greenhouse on 6 November (also Table III B).
F. Grown in a heated greenhouse with 18 hrs. light daily from 31 August.

Treatment	Late	September	October		November	December
	August		Early	Late		
<i>Leaflet Area (in sq. dm.)</i>						
A.	0.26	0.16	0.12	—	—	—
B.	0.27	0.23	0.25	0.21	—	—
C.	—	0.25	0.23	0.28	0.28	0.22
D.	—	0.13	0.17	0.20	0.22	0.29
E.	—	—	0.13	—	0.13	0.35
F.	0.30	0.23	0.19	0.23	0.22	0.34
<i>Number of cells per leaflet (in millions)</i>						
A.	2.29	1.22	1.02	—	—	—
B.	1.66	2.07	2.20	1.86	—	—
C.	—	1.87	1.81	1.95	2.37	2.47
D.	—	1.18	0.86	1.23	1.75	1.83
E.	—	—	0.79	—	1.05	1.86
<i>Epidermal cell area (in μ^2)</i>						
A.	1,130	1,340	1,170	—	—	—
B.	1,620	1,110	1,130	1,140	—	—
C.	—	1,340	1,270	1,420	1,170	890
D.	—	1,140	2,030	1,650	1,250	1,600
E.	—	—	1,950	—	1,270	1,890

at a later stage in the autumn. The plants which were put into long days early in October (*D*) were prevented from becoming inactive (which would have been indicated by lengthening of the plastochron interval), but they had already produced some smaller leaves; this latter trend was reversed by long-day treatment. But the plants which had been kept under natural conditions until early November had already almost ceased to produce fresh leaves, and the transfer to long days markedly increased the rate of leaf production as well as increasing leaf size. A similar effect is shown in Table II, but at a higher temperature range. These plants were kept in a heated greenhouse under natural autumn and winter daylengths, and became as inactive as normal

plants growing outside (Arney, 1955), and few plants produced even a single leaf each month. Long-day treatment commencing on 21 November induced rapid leaf production and a much enhanced leaf size, just as it does when applied to normally dormant plants.

Thus plants with a variety of prehistories all respond similarly to long-day treatment, through increased leaf size. The assumption that the change in leaf size is really a direct effect of daylength would be strengthened by a

TABLE II

The Effect of Photoperiod in a Heated Greenhouse on Leaf Structure

Plants were kept in a heated greenhouse under natural autumn short-day conditions; some of these plants were subjected to long days (18 hrs. light) from 21 November onwards.

Treatment	October	November	December	January
<i>Leaflet area (in sq. dm.)</i>				
Short-day throughout	0.13	0.10	0.11	0.11
Short-day into long-day	0.11	0.11	0.17	0.20
<i>Number of cells per leaflet (in millions)</i>				
Short-day throughout	1.14	0.78	0.76	0.73
Short-day into long-day	0.97	0.94	1.15	1.54
<i>Epidermal cell area (in μ^2)</i>				
Short-day throughout	1,180	1,250	1,350	1,510
Short-day into long-day	1,210	1,180	1,480	1,330

demonstration that the change from long days to short days produces the opposite effect. This evidence is given in Table III, showing a decrease in leaf size after transfer from long days back to short days, in three separate sets of plants with different prehistories. Unfortunately the effect could not be followed to completion because the short-day treatment consisted of the natural daylength of the season, and these daylengths were rapidly increasing by the end of February. However, there is no doubt that even this degree of short-day treatment has resulted in a reduction of leaf size in all three cases, the effect being significant almost at the 0.1 per cent. level.

The consistent effect of day length on leaflet size in these experiments confirms the results obtained by Borthwick and Parker (1953) with six other varieties of strawberry. They also used young runner plants in September and October, and obtained maximum leaf areas in 17 and 20 hours light daily; these *leaf* areas are just three times the *leaflet* areas given in Table I B, C, and F, for 18 hour photoperiods, so that there is complete agreement between the two sets of results. But the maximum leaf size achieved in any

of these experiments is somewhat less than half the size of the large early summer leaves produced by mature plants. This difference may be due to the immaturity of the young runner plants, which would not normally have produced large leaves until the succeeding summer, many plastochrons beyond the latest stage reached in these experiments. The possible effect of the more extensive root system of the maturer plants must not be overlooked, either.

TABLE III

Effect of Transfer to Short Days on Leaf Size, Cell Division, and Vacuolation

All plants were transferred to short days on 7 January without any change in temperature conditions. Previous history of these plants:

A. In long-day, heated greenhouse since 1 September.

B. Normal plants transferred to a long-day, heated greenhouse during October and November.

C. Plants kept in a short-day, heated greenhouse from September onwards, and transferred to the long-day, heated greenhouse on 21 November.

	December	January		February	March
		Early	Late		
<i>Leaflet Area (in sq. dm.)</i>					
A.	0.28	0.22	0.16	0.17	—
B.	0.32	0.38	0.26	0.24	0.14
C.	0.21	0.25	0.20	0.13	—
Weighted mean	0.28	0.31	0.22	0.21	—
<i>Number of cells per leaflet (in millions)</i>					
A.	1.78	1.61	1.12	1.15	—
B.	1.87	2.15	1.54	1.58	1.30
C.	1.32	1.69	1.59	0.79	—
Weighted mean	1.67	1.93	1.45	1.38	—
<i>Epidermal cell area (in μ^2)</i>					
A.	1,570	1,400	1,400	1,490	—
B.	1,770	1,770	1,710	1,540	1,070
C.	1,570	1,480	1,260	1,620	—
Weighted mean	1,680	1,610	1,510	1,540	—

Leaf size in these experiments continues to increase for two or three months after the first application of long days, and this points to a steadily mounting leaf-size potential, either as a result of more active growth and an altered hormone status, or of greater maturity. Defoliation experiments indicate that the number of mature functional leaves has a considerable effect on the size of expanding leaves, and the matter will be taken further in a future paper.

THE RELATIVE EFFECTS OF INTENSITY AND DURATION OF THE
SUPPLEMENTAL LIGHT DOSE

Preliminary experiments on the forcing of strawberry plants during the winter months indicated that possibly the intensity of daylight in the green-

house might be insufficient for active growth. The effect of long days on leaf size might therefore be due to the increased dose of light rather than to the longer duration of the daily light period. To resolve this ambiguity some short-day series were grown in high light intensities by supplementing daylight with artificial light throughout the whole daylight period. In some cases fluorescent lamps were switched on at 8.30 a.m. and switched off at 4.30 p.m. daily, thus giving an extra light intensity of 1,600 lux in addition to daylight, throughout most of the daylight period; in other series H.P.M.V. lamps were used to

TABLE IV

The Effect of Light Intensity on Leaf Size, Cell Division, and Vacuolation

Plants were grown in a heated greenhouse under natural winter daylengths. The light-intensity treatment consisted of 8–10 hrs. illumination by either 'daylight' fluorescent tubes or H.P.M.V. lamps, in addition to daylight, between dawn and dusk daily from 6 January onwards; there was no prolongation of the natural daylength.

	October	November	December	January	February	March
<i>Leaflet area (in sq. dm.)</i>						
Normal daylight	0.13	0.10	0.11	0.11	0.14	0.13
Increased intensity	0.11	0.08	0.09	0.09	0.11	0.12
<i>Number of cells per leaflet (in millions)</i>						
Normal daylight	1.14	0.78	0.76	0.73	0.87	1.16
Increased intensity	0.93	0.67	0.72	0.65	0.78	0.99
<i>Epidermal cell area (in μ^2)</i>						
Normal daylight	1,180	1,250	1,350	1,510	1,610	1,160
Increased intensity	1,150	1,170	1,280	1,320	1,460	1,240

produce an extra 2,000 lux for 10 hours each day, in addition to, and simultaneously with the winter daylight.

When plants which had previously been kept in short days in a heated greenhouse were given this additional light-intensity short-day treatment, there was no effect whatever on leaf size or cell number (Table IV), so that the effect of long days shown in Table II must be due to the lengthened light period and not to the increased dose of light. Normal plants brought from outside into a warm lighted greenhouse did show a temporary increase in leaf size as a result of the additional light-intensity short-day treatment, compared with the daylight alone (Table V, *B*, cf. *C* and *D*); the difference for early February was significant at the 0.1 per cent. level. Long days, however, produce a much greater increase in leaf size (Table V, *E*) than do the high light-intensity short days, and there can be no doubt that the length of the light period is more effective than the intensity of light in determining leaf size. The change from normal outside conditions to the heated greenhouse

under high light-intensity short-day treatment forced plants into activity almost as successfully as the long-day treatment, so that it seems feasible that the temporary increase in leaf size under high light-intensity short days shown in Table V may be a secondary effect of the forcing action of this treatment, and that, as indicated in Table IV, there is no direct effect of the higher light intensity on leaf structure.

TABLE V

Effect of Short-Day Forcing on Leaf Size, Cell Division, and Vacuolation

- A. Plants kept in a short-day, heated greenhouse throughout autumn and winter.
B. Normal plants brought from outside into the short-day, heated greenhouse, without any additional illumination, on 7 January.
C. Normal plants brought from outside into the short-day, heated greenhouse on January 7 with light intensity boosted by fluorescent lamps.
D. Normal plants brought from outside into the short-day, heated greenhouse on 7 January with light intensity boosted by H.P.M.V. lamps.
E. Normal plants brought from outside into the heated greenhouse and long days.

	October- November	January		February		March
		Early	Late	Early	Late	
<i>Leaflet area (in sq. dm.)</i>						
A.	0.13		0.11	0.13	0.15	0.13
B.	0.13		0.16	0.18	0.32	0.24
C.	0.13	0.19	0.22	0.26	0.33	0.21
D.	0.15	0.18	0.20	0.24	0.26	0.19
E.	—	—	0.23	0.47	0.55	—
<i>Number of cells per leaflet (in millions)</i>						
A.	1.14		0.73	0.71	1.07	1.16
B.	1.15		1.15	1.26	2.29	1.81
C.	1.03	1.10	1.33	1.64	2.31	1.62
D.	1.27	1.28	1.40	1.76	1.66	1.62
E.	—	—	1.10	2.30	3.30	—
<i>Epidermal cell area (in μ^2)</i>						
A.	1,180		1,510	1,840	1,400	1,160
B.	1,170		1,390	1,430	1,420	1,360
C.	1,210	1,660	1,690	1,570	1,430	1,290
D.	1,200	1,480	1,410	1,400	1,560	1,170
E.	—	—	2,100	2,020	1,670	—

An increased leaf size, and cell size, in high light intensities has previously been recorded for *Helianthus* (Penfound, 1932) and for *Acer platanoides* and *Cornus florida* (Isanogle, 1944). The absence of any general effect of light intensity on leaf size and structure in the present experiments may be due to the range of intensities involved, although it should be remembered that this range included an intensity which was barely sufficient for vigorous growth, and also intensities almost double this value. The results do, however, give a clear answer on the point for which they were intended, and demonstrate that it is the duration and not the total dose of light which is responsible for

the observed effect of daylength on leaf size. This is confirmed by the fact that plants exposed to 45 minutes of mercury vapour light (2,000 lux) between 1.15 and 2.0 at night in combination with the short days of autumn show an effect of the additional light which is out of all proportion to the additional dose of light involved (see Fig. 1, p. 76). The fact that this treatment is not as effective as prolonging the daylength to 18 hours, is probably associated with the relative ineffectiveness of mercury vapour light for the photoperiodic stimulation of strawberry plants. Roodenburg (1954) states that natural short days can be transformed into effective long days by giving substantial additional doses of red light *during the short light period*—i.e. by increasing the intensity rather than the duration of irradiation. Roodenburg interprets these, and other results, as showing that a shorter minimum period of red light than of blue light will act as long day; also that there is a rather high minimum intensity of red light for photoperiodic stimulation in certain plants, including the strawberry, which is not always obtained in natural daylight throughout the whole of the day. If this interpretation is correct, Roodenburg's results would not necessarily conflict with the data of Table IV, since the latter were obtained using blue-green light for supplementation. More detailed discussion of this aspect is premature at present.

CELL SIZE AND NUMBER OF CELLS PER LEAFLET

The tables show that most of the changes in leaf size are brought about by differences in the number of cells in each leaflet. The normal seasonal change in leaf size was also found to depend almost entirely on differences in cell number (Arney, 1954), and reasons were given for believing that these differences in cell number arose from different durations of the cell division phase relative to the stage of leaf development, and not to differences in the rate of cell division based on the plastochron as a unit of time. Determinations on emergent leaves from a number of different treatments show that the number of cells per leaflet at the emergent stage is approximately constant, regardless of the final size and cell number which will be attained at maturity. Thus, differences in mature leaf structure, as regards cell size and number of cells per leaf, are acquired during expansion of the leaflet *after* emergence; the effect of photoperiod is clearly shown to be operative through the prolongation of the cell-division phase after emergence.

There are very few statistically significant differences in mature cell size between any of the various treatments given in 1953-4. The gradual increase in cell size throughout the late autumn and early winter under short days in the heated greenhouse is significant at the 1 per cent. level, and is not altered by transfer to long days (Tables II and IV). This seasonal effect may be a reflection of the decreased water tension in the plant as the large, older, summer leaves die off. There is no clear-cut effect of high light intensity on cell size; the consistently smaller cell size of the high light-intensity short-day leaves (Table IV) is not statistically significant. An opposite difference is seen in Table V, which shows a tendency towards larger cells immediately after

the application of the high light-intensity short-day treatment *C* in January and February; but the differences are barely significant at the 5 per cent. level even. Taken as a whole, these results do not give evidence for an effect of increased light intensity on cell size, as in the investigations quoted by Shields (1950).

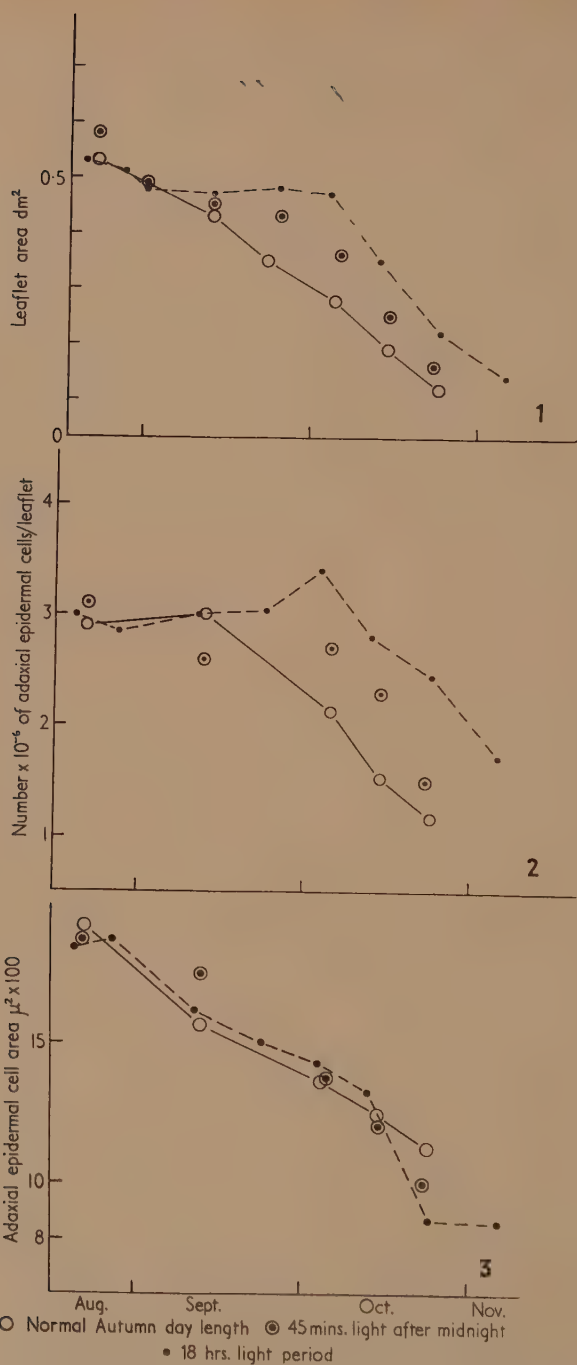
Figs. 1, 2, and 3 show the results obtained with plants growing out of doors during the autumn and winter of 1954-5, under mercury vapour lamps as a source of supplemental illumination to give 18 hours light daily. Long days have a marked effect on both leaf size and cell number; the latter does not fall below the August value until the end of October, and these leaves would not have completed their cell division phase until after the first week in November, by which time the minimum night temperature was consistently below 40° F. The cell size and cell number of the leaves in this series under mercury vapour lights are much higher than in the 1953-4 series (Table I). This difference is not caused by the mercury vapour lights, but by a very high level of N.P.K. manuring, and will be discussed in a subsequent paper. These values should be regarded as abnormally high; the 1953-4 values were not subnormal.

THE EFFECT OF TEMPERATURE ON LEAF SIZE AND CELL SIZE

No facilities were available for growing comparable series of plants at different constant temperature levels, and only a few tentative considerations can be advanced on the basis of the data available. Temperature does not seem to exert a predominant influence on leaf size over the normal cultural range between 45° and 70° F. There is very little difference in leaf size, for example, between treatments *B* and *F* in Table I, or between *B* and *C* of this same table. In both cases the plants in the greenhouse were growing at temperatures which were over 10° higher than those in the open. The close similarity in leaf size of plants grown under natural autumn daylengths in the greenhouse (Table IV), and outside (Table I, *A*) shows that the normal decrease in leaf size during the autumn can be induced by short days alone, without any fall in temperature.

But there is an indication that leaf size may decrease slightly as winter approaches under long day treatment also (Table I, *A*). A similar series in 1954-5 using H.P.M.V. lamps has again shown a decrease in size of leaves expanding in November and December in long days. A consideration of daily records of the maximum temperatures during both seasons suggests that leaf size is appreciably reduced, even in long days, when the daily temperature range falls below the 40°-50° range; but on the basis of only two seasons' observations these conclusions must be very tentative, and it is impossible to decide whether day or night temperature is the more important.

The effect of temperature on mature cell size is shown in Fig. 3. The marked decline in mature cell size during the autumn is exactly the same under all photoperiodic treatments, and cannot be ascribed to the changing photoperiod of autumn. Evidence has already been mentioned that light intensity does not materially affect cell size under autumn and winter conditions; and the long-



FIGS. 1-3. Effects of photoperiod on autumn leaf size (FIG. 1), cell number per leaflet (FIG. 2) and epidermal cell size (FIG. 3).

day plants in Fig. 3 remained actively growing throughout the period of decline in cell size, so that the decrease in cell size cannot be ascribed to the onset of dormancy. A more exact analysis of these temperature effects must await facilities for growing plants at a number of selected and controlled temperature ranges. It must be emphasized that the *rate* of cell division, as opposed to the duration of the cell-division phase, is more closely related to temperature (Arney, 1954); by using the plastochron as a measure of time in determining the duration of the cell-division phase, this effect of temperature on rate of cell division can largely be discounted in considering the ontogenetic duration of the cell-division phase.

THE RELATION BETWEEN CELL DIVISION AND VACUOLATION

It has previously been pointed out (Ashby, 1950; Arney, 1954) that there is sometimes a tendency for the cell size of leaves to be inversely related to the number of cells per leaf, and this has been interpreted to mean that unusually prolonged periods of cell division may leave insufficient time for the subsequent vacuolation of the daughter cells. The method of determination of cell size and cell number can generate a spurious negative correlation between these two quantities since both are calculated from the same primary determination—the number of cells occupying a given small area of tissue. Such a spurious correlation is unlikely to be responsible for an appreciable proportion of a statistically significant correlation if the percentage error in determination of cell size is small compared with the variation in cell size over which the correlation is to be established. The negative correlations reported previously (Arney, 1954) for strawberry leaves can be regarded as being only slightly invalidated in this respect. It seems likely that the same is true of Ashby's conclusions, although the published data is not sufficient to be certain of this.

In the present series of experiments about one-third of all the sets of replicates showed a statistically significant negative correlation between cell area and cell number within the batch of replicates for any one occasion and treatment. But in only two of these cases (for January, Table IV) was the variation within the sample sufficiently great to make it certain that the spurious correlation could not be preponderant. The mean values for cell area and cell number (Tables I–V) show no correlation, either within each treatment separately, or over the whole set of experimental results. This absence of correlation over a considerable range of leaf size shows that the prolonged periods of cell division which occurred in certain of the treatments did not involve any subsequent reduction in the vacuolation of the cells. This confirms the previous suggestion (Arney, 1954) that it is only near the maximum obtainable leaf size and cell number for the genotype that prolonged cell division curtails vacuolation and reduces the final size of the cells.

In a stimulating essay on morphogenesis, Bonner (1952) points out that Sinott's investigations on cucurbit fruits showed the same logarithmic increase in size over both the cell division and vacuolation phases of growth, and proceeds to make the generalization: 'since both cell multiplication and cell

enlargement constitute growth, there is no discrimination between them, but they both increase with increased size.' This generalization will not fit the results with strawberry leaves, for the final size depends almost entirely upon the duration of the cell-division phase of growth. Moreover, the elongation rate of the leaf initial after emergence is much higher than would have been the case if the previous logarithmic increase in elongation rate had continued, and the growth after emergence proceeds by successive daily increments in length which are all equal, so that growth is no longer obeying a logarithmic law. It is now clear that cell enlargement—'vacuolation'—is not merely the absorption of water by cells, but is a phase in which there is very considerable protoplasmic growth (Morgan and Reith, 1954). Nevertheless it is still important to distinguish between cell division and cell enlargement as distinct growth processes, since the latter does not involve an increase in nuclear material, and by its very nature must be limited in extent.

CONCLUSIONS

1. Long days have the effect of prolonging the period of cell division in the expanding leaf, while short days curtail cell division. In short days (10 hours light) cell division stops shortly after emergence, and many cells do not divide again after the leaf has emerged. Leaf size is correspondingly affected by photoperiod.

2. Changes in temperature between 45° and 70° F. have little effect on number of cells per leaf, or on leaf size. Temperatures below 45° F. result in smaller cells and leaves, and at still lower temperatures the duration of cell division is curtailed.

3. The normal seasonal change in leaf size is shown to be mainly due to photoperiod, but with temperature playing an important part in early spring and late autumn.

4. Leaflet size, and number of cells per leaflet at the stage of emergence are rather variable, even within each treatment; there are no statistically significant differences in leaflet size or cell number at emergence between the treatments so far investigated.

5. There is no evidence that the degree of vacuolation of the epidermal cells is affected by the duration of the cell-division phase under the conditions obtaining in these experiments, in which the number of cells per leaf was not at the maximum for the variety.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the generosity of University College, Cardiff, in making a special research grant to cover the cost of research assistance, and to thank Mr. John Clark for his painstaking and methodical accomplishment of the many cell counts involved.

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The Nature of the Resistance of Oats to the Take-all Fungus

II. INHIBITION OF GROWTH AND RESPIRATION OF *OPHIOBOLUS GRAMINIS* SACC. AND OTHER FUNGI BY A CONSTITUENT OF OAT SAP

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Received 17 May 1955

SUMMARY

Growth of *Ophiobolus graminis* and of *O. graminis* var. *avenae* is inhibited by concentrations of 3.3–4.0 $\mu\text{g.}/\text{ml.}$, and respiration by concentrations of 55 $\mu\text{g.}/\text{ml.}$ of a partially purified substance from oat-leaf sap. The two varieties appear to be equally sensitive. The filtrate of boiled sap is inhibitory but here dilution of the sap permits better growth of isolates of var. *avenae*. Sap from oat roots is inhibitory to *O. graminis* only, and fractionation of the sap shows that the inhibitor can be masked by a growth stimulant. Inhibition of growth and respiration can be reduced by glutathione and ascorbic acid, particularly if the inhibitor and reducing agent are previously incubated together for a few hours, suggesting that the inhibitor is inactivated on reduction. The capacity of var. *avenae* to overcome inhibition in the favourable medium provided by the crude sap more readily than can the type variety is suggested as the cause of the slight differential activity of the filtrate of leaf sap and the full differential activity of the root sap. Susceptibility of oats to var. *avenae* would thus be due to conditions enabling the fungus to overcome toxicity rather than to an absence of toxicity.

Activity of the inhibitor against growth and respiration of a number of fungi and a few other organisms has been tested. Bacteria and oat and barley roots are not affected but about half of the fungi tested are inhibited although none is as sensitive as *O. graminis*. No members of the fungi imperfecti tested are sensitive.

INTRODUCTION

A DIFFERENTIAL inhibitory action of sap from oat roots has been described whereby *Ophiobolus graminis* Sacc. (the fungal pathogen causing take-all of wheat and barley) is killed but the closely related *O. graminis* var. *avenae*, capable of also attacking oats, is not (Turner, 1952). It was suggested that the resistance of oats to the former and its susceptibility to the latter can be correlated with this effect. A substance crudely purified from leaf sap was found to be strongly inhibitory to *O. graminis* and it was suggested that this contained the differential inhibitor. Further investigation has shown, however, that in a more highly purified state, this substance is inhibitory to both

varieties. The reaction of both varieties to this material and its action as an inhibitor of growth and respiration in more than half of a range of fungi examined are described below.

MATERIALS AND METHODS

Preparation of inhibitor. An aqueous extract of minced leaves from oat seedlings (Victory), 3 to 5 weeks old, is boiled and filtered. The coagulum on to which much of the active material is adsorbed, is washed several times with ether and ethanol and extracted with methanol. This extract is washed as before and the almost colourless residue is stored in a desiccator. Under these conditions the material remains active for at least 4 months. It is slightly soluble in methanol (0.2 mg./ml.) and very slightly soluble in water (0.05 mg./ml.).

The filtrate obtained in the first stage of this preparation is also strongly active. Its action is similar to that of the semi-pure inhibitor, and it was used in some experiments comparing the reaction of different fungal isolates which are described below.

An active juice from oat roots is obtained by extracting the roots with water and washing the extract with benzene and chloroform. This crude extract is toxic to *O. graminis* but not to var. *avenae*.

Origin and character of isolates of O. graminis and O. graminis var. avenae. Eleven isolates were used in all, of which six were isolated from wheat, one from barley, three from oats, and one from *Agrostis* sps. These were designated W or O according to ascospore measurement, a spore length of 68–104 μ with average lengths for single isolates varying from 79 to 86 μ being previously described as characteristic of *O. graminis* (W), and 84–130 μ with averages from 101 to 117 μ characteristic of var. *avenae* (O) (Turner, 1940). Details of the isolates used are given in Table I.

TABLE I

Isolate	Host	Collected by	Date and place of collection	Spore length	
				average	range
W	Winter wheat	Dr. R. Lucas	1952 Oxfordshire	short	
W ₁	" "	Dr. Bradley Jones	1954 Glamorgan	88 μ	71–100 μ
W ₂	" "	" "	" "	81 μ	68–97 μ
W ₅	" barley	" "	" "	87 μ	74–97 μ
O ₁	<i>Agrostis</i> sps.	Mr. Drew Smith	1953 Yorkshire	long	
O ₂	Winter oats	Dr. Bradley Jones	1954 Glamorgan	100 μ	87–123 μ
O ₃	" "	" "	" "	104 μ	97–120 μ
O ₄	" "	" "	" "	114 μ	99–123 μ
O ₅	" wheat	" "	" "	105 μ	91–120 μ
O ₆	" "	" "	" "	105 μ	94–113 μ

In the present group of isolates all those designated O are much more profuse producers of microconidia than are the W isolates. The varieties show no other morphological differences, colour ranging from light to dark between isolates of each variety (cf. White, 1942).

Growth of fungi and assay of inhibitor. The minimal nutrient medium, modified from White (1941), has the following composition per litre:

MgSO₄ 1.0 g. (2.0 g. hydrated), KH₂PO₄ 1.0 g. Na₂HPO₄ 3.0 g. (8.0 g. hydrated), FeCl₃ trace, glucose 20 g., NH₄NO₃ 5.0 g., biotin 1 mg., aneurin 1 mg.

The glucose is added after autoclaving. Additions of other substances are made in certain experiments, thermolabile substances such as ascorbic acid being sterilized by filtration through sintered glass. Flasks of 100 ml. capacity are used, each containing 5 ml. of medium. Each is inoculated with a single disk 3 mm. in diameter cut with a drawn-out glass tube from the growing edge of mycelial mats on 2 per cent. malt agar. Four replicate flasks are prepared for each treatment. The flasks are incubated at 20° C. (maximum) for 5 days in the case of *O. graminis*, and varying periods for other fungi, and the growth made is then measured by taking the dry weight. A control is run for each experiment to ascertain growth in minimal nutrient alone, and results are generally expressed as a percentage of this.

All W isolates grow well on the minimal medium although growth is improved by addition of such substances as hydrolysed casein or yeast extract. Of the O isolates, O₁, O₃, and O₄ grow well but O₂, O₅, and O₆ make poor growth; these also grow more slowly in complex media such as malt, potato dextrose and wheat root extract and glucose. Flasks inoculated with these isolates are incubated for 9 or more days before the growth is measured.

EXPERIMENTAL

In earlier experiments only *O. graminis*, isolate W, was used. The full range of isolates was used later to compare reaction to the inhibitor of the two varieties. The two series of experiments will be described together where convenient.

Reaction of the two varieties to crude root juice. The differential reaction of the two varieties of *O. graminis* to sap from oat roots described in an earlier paper (Turner, 1952) is illustrated in Table II.

TABLE II

Growth of Isolates W and O₃ in Root Sap in 120 hours (mg.)

(Figures in brackets represent growth relative to control)

	Isolate W	Isolate O ₃
Control-minimal medium . . .	41.0 (100)	30.5 (100)
Wheat-root juice + 2% glucose . .	85.0 (208)	86.0 (282)
Oat-root juice + 2% glucose . . .	2.0 (5)	33.5 (110)

Dilution of oat-root sap improves growth of both isolates, growth of W improving slightly and of O₃ soon equalling that in wheat-root juice. Un-autoclaved juices have the same effects.

Activity of the semi-purified substance from oat leaves

(a) *Inhibition of growth of isolate W.* In Table III are given results of a representative experiment carried out to determine activity of a given preparation of inhibitor. Preparations made at different times vary in activity; a 50 per cent. reduction in growth is effected by concentrations ranging between 3.3 and 4.0 $\mu\text{g./ml.}$

TABLE III

Inhibition of Growth of Isolate W in Minimal Medium

Concentration of inhibitor in $\mu\text{g./ml.}$	Nil	5.0	4.75	4.5	4.25	4.0	3.75
Growth after 5 days { in mgm.	48	6	4	12	19	24	36
relative to control	100	13	9	25	39	50	75

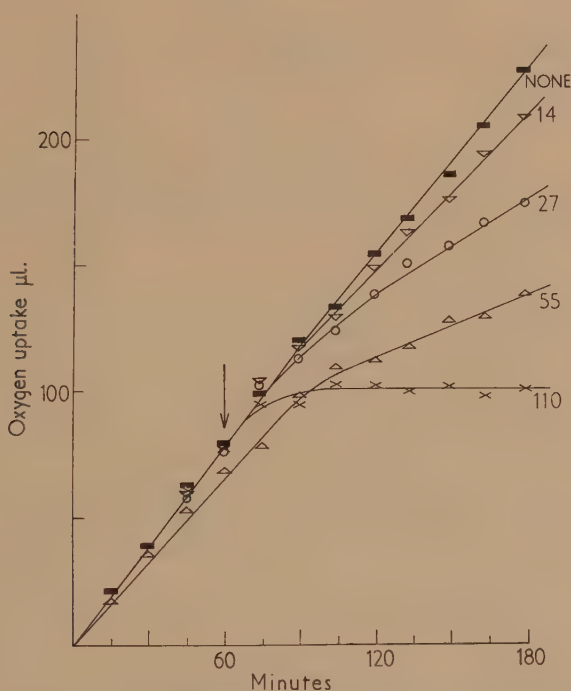


FIG. 1. Inhibition of respiration of isolate W by various inhibitor concentrations shown on curves as $\mu\text{g./ml.}$

(b) *Inhibition of respiration.* The effect of the inhibitor on the rate of respiration of the fungus was investigated using Warburg manometers. A reduction of 50 per cent. in oxygen uptake was effected by a concentration of about 55 $\mu\text{g./ml.}$ (Fig. 1), about fourteen times the concentration required to reduce growth to the same extent.

(c) *Comparison of different isolates.* The growth of nine isolates with different concentrations of inhibitor is shown in Fig. 2, and the effect of the inhibitor

on respiration of different isolates in Table IV, where are grouped the results of three experiments using different preparations of inhibitor.

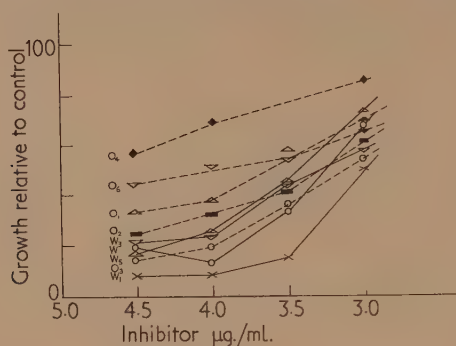


FIG. 2. Growth of W and O isolates in various concentrations of inhibitor

TABLE IV

Inhibition of Respiration of Various Isolates

The oxygen uptake in one hour before adding inhibitor is taken as unity and the figures represent the uptake relative to this in the second hour after addition.

		Concentration of inhibitor in µg./ml.									
		W	W ₁	W ₃	W ₅	O ₁	O ₂	O ₃	O ₄	O ₅	O ₆
Experiment 1	55	—	0.08	0.13	0.13	—	0.07	0.21	0.22	—	—
	83	0.58	0.61	—	—	0.53	—	—	—	—	0.65
" 2	55	0.87	0.81	—	—	—	0.84	—	—	—	—
	83	0.40	—	0.43	0.40	0.35	—	—	0.43	0.43	—
" 3	55	—	—	0.47	0.42	0.37	—	—	0.64	0.56	—

Reaction to the semi-pure substance from leaf sap therefore does not show the same clear difference between the two varieties as is found when crude sap from the roots is used. Growth of all W isolates is strongly inhibited. The faster growing O isolates are also sensitive to the inhibitor although O₁ makes slightly more growth in the presence of the higher concentrations, and O₄ makes better growth throughout. Of the slower growing isolates, O₂ shows the same pattern of response as do the W isolates; O₆ is less affected, but this may be simply a reflection of its inability to make good growth in the minimal medium. Respiration of all isolates is inhibited, but variation in degree of inhibition within varieties is high; O₄ again is slightly less sensitive.

The filtrate obtained from the boiled sap from oat leaves shows a more differential toxicity than does the purified material, being less toxic to O than to W isolates particularly at greater dilutions (see Fig. 3). When the sap is too dilute to be inhibitory it provides a good medium for growth, as is shown by the growth of O₃.

The strongly differential activity of the crude root sap is thus more faintly in evidence in the case of clarified leaf sap and only remains as a variable

concentration effect in the purified material. This suggests either that two inhibitors are present, one of which, obtained on purification, has little differential action, or that the activity of the toxin, or the reaction of the O isolates to it, is modified by other substances present in the crude sap.

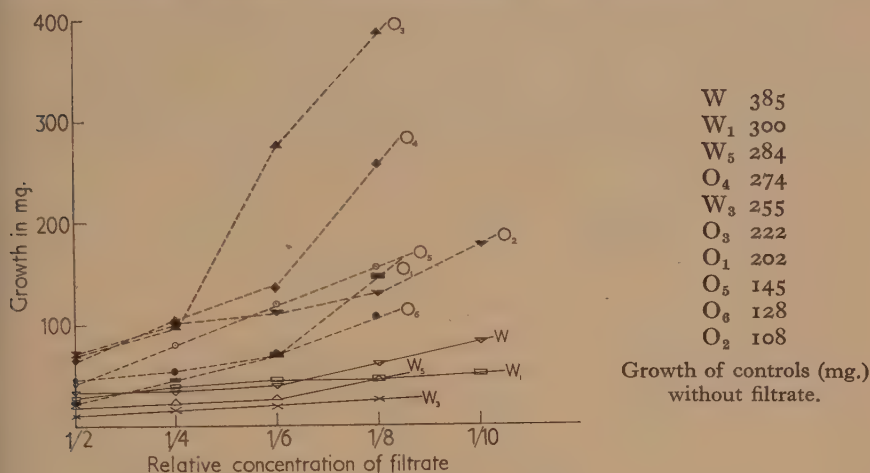


FIG. 3. Growth of W and O isolates in filtrate of boiled oat-leaf sap at various dilutions

Table V shows the results of a rough fractionation of oat-root juice. On centrifuging the juice, a clear brown supernatant liquor is obtained which stimulates growth of isolate W. When this is dried and extracted with methanol and then water, the growth stimulants are obtained in the former fraction. No toxicity is evident. The methanol extract of the residue of centrifugation also stimulates growth but in this case dilution leads to further stimulation of growth. Extraction of the residue with water after methanol yields further stimulatory substances but dilution reduces stimulation suggesting that there is no toxic substance in this fraction.

TABLE V

Effect of Different Fractions of Centrifuged Oat-Root Juice on Growth of Isolate W

Additions to minimal medium	Growth relative to control
None	100
Supernatant liquor equal vol.	240
Methanol soluble fraction of supernatant 1 mg./ml.	320
Methanol insoluble fraction. 4 mg./ml.	118
Methanol extract of residue of centrifugation 0.4 mg./ml.	144
0.2 mg./ml.	148
0.13 mg./ml.	160
0.1 mg./ml.	205
Water extract of methanol extracted residue 0.4 mg./ml.	192
0.1 mg./ml.	140

The methanol extract of the residue seems therefore to contain a growth stimulant, the effect of which is masked at the higher concentrations by an inhibitor. Purification of the inhibitor from this source has not been proceeded with, but possible masking or alleviation of toxicity was examined further.

Alleviation of toxicity. Inhibition of growth is most severe when the minimal nutrient is used, or when low concentrations (0.05 per cent. or less) of complex nitrogenous compounds such as hydrolysed casein are added. Addition of peptone or yeast extract increases growth in the presence of the toxin but, as shown in Fig. 4, the response to these two substances is different.

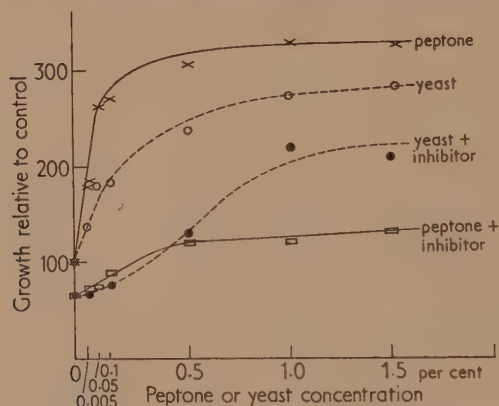


FIG. 4. Effect of additions of yeast and peptone on toxicity to isolate W

Peptone increases growth up to a low level, reached with an addition of 0.1 per cent. peptone, after which further addition of peptone has no effect, whereas increasing concentrations of yeast extract result in increasing amounts of growth until, with an addition of 1 per cent. yeast extract, growth rate approaches that in the same medium without inhibitor.

An examination of some constituents of yeast extract (vitamins and amino-acids) showed alleviation of toxicity by one amino-acid fraction (see Table VI). The constituent amino-acids of this fraction, cysteine, glutamic acid and valine, were tested individually at a concentration of 0.004M, as also was glutathione, and Table VII shows that cysteine and glutathione reduce inhibition.

Table VIII shows protection against growth inhibition of 6 isolates by glutathione.

The effect of glutathione on inhibition of respiration of isolate W was examined and the results are given in Table IX.

A direct effect of glutathione on the inhibitor is thus indicated. Table X shows that preincubation of inhibitor with glutathione over night increases the protective effect of the glutathione on several isolates.

Similar experiments were carried out using ascorbic acid instead of glutathione and the results given in Tables X and XII indicate that the protective action is due to the effect of reducing agents on the inhibitor.

TABLE VI

Inhibition of Growth of Isolate W in the Presence of Certain Constituents of Yeast Extract

The B vitamin mixture contained the following: *p*-aminobenzoic acid 80 μ g., riboflavine 43 μ g., pantothenic acid 200 μ g., nicotinic acid 182 μ g., pyridoxine 22 μ g. in 10 ml. This was diluted $\times 10$ for use.

The amino-acid mixture was as given by Block and Bolling, 1951, with some modifications. It was made up in 4 fractions which were used together or separately. These fractions were as follows: Fraction I—cysteine 11 mg., valine 70 mg, glutamate 147 mg.; fraction II—methionine 20 mg., histidine 30 mg., lysine 70 mg.; fraction III—tyrosine 36 mg., tryptophane 13 mg., phenylalanine 45 mg.; fraction IV—cystine 11 mg., threonine 55 mg., leucine 75 mg., isoleucine 60 mg. Each fraction was made up in 12.5 ml. for final dilution 8 times to 100 ml. so that the concentration of each amino-acid in the mixture was roughly equal to its concentration in a 1% solution of yeast extract.

Addition to minimal nutrient	Relative growth	
	Without toxin	With toxin
None	100	33
B vitamins equivalent to 1% yeast extract	104	31
Amino acids	111	42
Amino-acid fraction I	131	73
" " " II	72	41
" " " III	67	31
" " " IV	77	26
Yeast extract 1%	261	111

TABLE VII

Effect of Constituents of Amino-Acid Fraction I and of Glutathione

Addition to minimal nutrient	Relative growth	
	Without toxin	With toxin
None	100	37
Glutamate 0.004 M	138	43
Valine 0.004 M	100	24
Cysteine 0.004 M	138	50
Glutathione 0.002 M	115	63

TABLE VIII

Effect of Glutathione on Inhibition of Growth

Addition to minimal medium	Relative growth of isolate				
	W	W ₁	W ₃	O ₁	O ₂
None	100	100	100	100	100
Inhibitor at 4 μ g./ml.	53	23	46	49	36
Inhibitor at 4 μ g./ml. + 0.002 M glutathione	64	28	58	58	46
Inhibitor at 3.7 μ g./ml.	68	35	74	67	53

TABLE IX

The Effect of Glutathione on Inhibition of Respiration of Isolate W

The experiment was run for 3 hours, the contents of the side arm being added to the flask after 1 hour; steady values were reached 1 hour after this.

Final concentration of glutathione was 1 mg./ml. and of inhibitor 55 μ g./ml. throughout.

Addition to manometer flask	Addition from side arm	(a) Oxygen uptake in 1st hour	(b) Oxygen uptake in 3rd hour	b/a
None	None	127	135	1.06
None	Glutathione	155	158	1.02
None	Inhibitor	150	27	0.18
Glutathione	Inhibitor	132	44	0.33
Inhibitor	Glutathione	53	46	0.82
None	Inhib: + glut:	140	78	0.56

TABLE X

Effect of Glutathione on Inhibition of Respiration of Several Isolates

Oxygen uptake in 1 hour before additions = 1.0

Addition	Isolate					
	W ₁	W ₃	W ₅	O ₂	O ₃	O ₄
Nil	—	1.10	1.07	1.11	—	1.02
Glutathione 2 mg./ml.	1.11	—	—	0.93	0.81	0.75
Inhibitor 55 μ g./ml.	0.08	0.13	0.13	0.07	0.21	0.22
Inhibitor + glutathione	0.17	0.21	0.52	0.13	0.36	0.30
Inhibitor + glutathione preincubated	0.67	0.40	0.85	0.38	0.89	—

TABLE XI

Effect of Ascorbic Acid on Inhibition of Growth of Isolate W

Addition to minimal medium						Relative growth
None	100
Inhibitor at 4 μ g./ml.	16
"	"	"	+ ascorbic acid at 0.001 M			27
"	"	"	"	"	0.002 M	50
"	"	"	"	"	0.004 M	79

TABLE XII

Effect of Ascorbic Acid on Inhibition of Respiration of Three Isolates

Oxygen uptake before additions = 1.0

Additions	Isolate		
	W	O ₁	O ₆
None	1.09	—	—
Ascorbic acid 2 mg./ml.	1.12	0.92	1.06
Inhibitor 55 μ g./ml.	0.46	0.41	0.56
Inhibitor + ascorbic acid	0.67	0.62	0.76
Inhibitor + ascorbic acid preincubated	0.96	0.92	0.89

Ascorbic acid added to the sap from oat roots completely neutralizes its inhibitory action and results in excellent conditions for growth of W isolates. Filtrate of boiled sap from oat leaves is much more strongly inhibitory, but here again conditions for growth of both varieties are improved by ascorbic acid.

TABLE XIII

Growth of Three Isolates in Filtrate of Boiled Leaf Juice and in Root Juice with and without Ascorbic Acid

Medium	Isolate		Growth in mg.					
	Ascorbic acid concentration	Dilution	W		W ₁		O ₃	
			nil	0.004M	nil	0.004M	nil	0.004M
Root juice + 1% glucose	.	.	37	125	—	—	96	105
Leaf juice	1:2	.	2	3	—	—	6	10
+minimal medium	1:4	.	4	6	—	—		
" "	1:8	.	8	44	9	44	18	32
Minimal medium	.	.	51	62	54	61	21	28

INHIBITION OF GROWTH AND RESPIRATION OF OTHER FUNGI

A variety of fungi and a few other organisms were tested for susceptibility to the inhibitor. The species tested and the growth and rate of respiration with inhibitor expressed as a percentage of those without inhibitor are listed in Table XIV. The fungi were all grown in minimal nutrient plus 0.5 per cent. peptone. The bacteria were grown in seeded plates of peptone agar with inhibitor in punched holes. Respiration rate of roots of oats and barley was measured in phosphate buffer.

Growth of the two bacteria examined and respiration of oat and barley roots were not affected by the inhibitor. Of the fungi examined an inhibition of growth occurred in about half. In all cases in which both growth and respiration rate were examined, inhibition of one was accompanied by inhibition of the other with the exception of *Trichoderma viride* which showed a slight reduction in respiration rate but none in growth. In *Sordaria* sp. and *Neurospora crassa* rate of growth was reduced but final growth with inhibitor equalled that in absence of inhibitor. In all other cases inhibition of growth was permanent.

With the exception of the fungi imperfecti there appears to be no connexion between taxonomic position and susceptibility, different species of the same genus in one case (*Mucor*) reacting differently. None of the fungi imperfecti tested is sensitive, with the possible exception of *Trichoderma viride*.

TABLE XIV

Species	Growth (concentration of inhibitor 10 µg./ml.)	Respiration rate (con- centration of inhibitor 100 µg./ml.)
(a) Phycomycetes		
<i>Mucor hiemalis</i> + . . .	63	68
" " — . . .	82	—
" <i>ramanianus</i> . . .	93	96
<i>Absidia glauca</i> + . . .	67	—
" " — . . .	77	—
" <i>butleri</i> + . . .	82	—
" " — . . .	88	—
<i>Phycomyces blakesleanus</i> + . . .	19	33
" " — . . .	25	—
<i>Cunninghamella elegans</i> + . . .	91	—
" " — . . .	91	—
<i>Thamnidium elegans</i> . . .	68	—
<i>Syncephalastrum racemosum</i> . . .	68	—
<i>Helicomyces pyriforme</i> . . .	23	—
<i>Circinella nigra</i> . . .	75	—
<i>Rhizopus nigricans</i> + . . .	102	—
" " — . . .	101	—
" sp. (homothallic) . . .	87	110
<i>Blakeslea trispora</i> . . .	107	—
<i>Pythium ultimum</i> . . .	97	—
<i>Sapromyces elongatus</i> . . .	70	—
<i>Allomyces arbuscula</i> . . .	40	—
(b) Ascomycetes		
<i>Saccharomyces cerevisiae</i> . . .	94 ¹	150
<i>Schizosaccharomyces octosporus</i> . . .	—	60
<i>Sordaria</i> sp. . . .	92 ¹	51
<i>Neurospora crassa</i> . . .	82 ^{1, 2}	—
<i>Monascus purpureus</i> . . .	15	53
<i>Glomerella cingulata</i> . . .	92	—
(c) Basidiomycetes		
<i>Coprinus fimetarius</i> . . .	28	59
<i>Polyporus betulinus</i> (dikaryotic) . . .	16	—
" " (monokaryotic) . . .	10	—
<i>Marasmius peronatus</i> . . .	82	—
<i>Hypholoma fasciculare</i> . . .	80	—
(d) Fungi imperfecti		
<i>Verticillium cinnabarinum</i> . . .	91 ¹	93
<i>Alternaria tenuis</i> . . .	91 ¹	92
<i>Fusarium culmorum</i> . . .	96	92
<i>Trichoderma viride</i> . . .	100	80
<i>Aspergillus niger</i> . . .	94 ¹	—
" <i>nidulans</i> . . .	98 ¹	—
<i>Penicillium</i> sp. . . .	104 ¹	—
" <i>spinulosum</i> . . .	97	—
<i>Helminthosporium gramineum</i> . . .	94	108
<i>Oospora lactis</i> . . .	102 ¹	—
<i>Stysanus stemonitis</i> . . .	95 ¹	—
<i>Pestalozzia</i> sp. . . .	99 ¹	—

¹ Growth measured as diameter of colonies on agar instead of as dry weight.² Growth delayed by inhibitor by 2 or 3 days.

TABLE XIV (cont.)

Species					Growth (concentration of inhibitor 10 µg./ml.)	Respiration rate (con- centration of inhibitor 100 µg./ml.)
(e) Bacteria						
<i>Staphylococcus aureus</i>	no zone of inhibition	—
<i>Escherichia coli</i>	" " " "	—
(f) Cereal roots						
Oats	—	89
Barley	—	104

DISCUSSION

The substance isolated, as described above, from oat-leaf sap is thermostable, destroyed on prolonged boiling with acid but not with alkali, and slightly soluble in methanol and in water. Its inhibitory action is stronger against *O. graminis* than against any other fungi tested but it is capable of inhibiting growth of about half the fungi tested at a concentration of 10 p.p.m. Respiration is inhibited by concentrations 10 to 20 times stronger than those required for growth inhibition. Of the organisms tested only fungi have been found to be sensitive, and of the fungi, only members of the fungi imperfecti show a general insensitivity, but the range of organisms is not wide enough for generalization to be made.

Both varieties of *O. graminis* are fully inhibited in growth and respiration by higher concentrations of the semi-pure material. Some isolates of var. *avenae* (O_1 and O_3) are as sensitive as are W isolates; O_4 shows greater insensitivity at lower concentrations, and the slower-growing isolates O_5 and O_6 are less reduced in growth compared to the control in minimal medium but growth is in any case very slow.

Considering the isolates of var. *avenae* as a group therefore, it cannot be said that they react differently from the W isolates; this is in contrast to the reaction of the two groups to oat-root juice.

The identity of the inhibitory substance in root juice with that purified from leaf juice is not certain. Activity of the root juice is very much less and it differentiates fully between the two varieties. The inhibitory substance here also is soluble in methanol and its activity is reduced by ascorbic acid. Growth-stimulating substances in the same methanol-soluble fraction, in which they are apparently present in greater proportional concentration than in the crude sap, prevent toxic action by this fraction, but since dilution increases growth stimulation and since the inhibitor does not reside in other fractions of the sap its presence here is assumed. Isolates of var. *avenae* do not suffer inhibition by the root sap; the concentration of inhibitor may be sufficiently low to allow these isolates to overcome its effects in the presence of the stimulatory or antagonistic substances also present in the sap. In this connexion it is significant that dilution of the filtrate of leaf sap, fully toxic to both varieties when undiluted, allows a much greater increase in growth

of var. *avenae* isolates than of the W isolates. The effect is therefore not a simple one of concentration of the pure substance, since both varieties are equally affected by this, but of its concentration in the favourable medium provided by the plant sap. The ameliorative effect of reducing agents also shows that the inhibitor can be neutralized at lower concentrations, although this has not in general been found to be more effective with the O than with the W isolates.

The existence of conditions in the sap of oat roots which enable var. *avenae* to overcome the inhibitor and so to parasitize the roots is therefore suggested. This is in agreement with the view that susceptibility rather than resistance is a positive condition, resulting from interaction of specific products or capacities of host and parasite (cf. Allen, 1954).

ACKNOWLEDGEMENTS

I am indebted to Mr. J. Drew Smith, of the St. Ives Research Station, Bingley, Yorks., and to Dr. J. Bradley Jones, of the National Agricultural Advisory Service (Wales), Cardiff, for kindly supplying me with material infected with *Ophiobolus graminis* and *O. graminis* var. *avenae* and for helpful information concerning distribution of the pathogens.

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The Action of Thiourea as a Germination Stimulator

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Received 9 February 1955

SUMMARY

It has been shown that thiourea is not metabolized by lettuce seeds during germination despite its stimulatory action. Thiourea was known to enter the seeds at much the same rate as water. Substitution of thiourea converted it from a stimulatory to a strongly inhibitory substance.

INTRODUCTION

THIOUREA has long been known as a dormancy-breaking substance (Denny *et al.*, 1930). Its mechanism of action as a dormancy-breaking substance is still entirely unknown (Thompson, 1953). Thiourea has been used in these as in many other laboratories as a dormancy-breaking substance for seeds. It is particularly useful in abolishing the light requirement of light-sensitive lettuce seeds, such as variety Grand Rapids.

A knowledge of whether thiourea is in fact metabolized in lettuce seeds seemed therefore imperative.

It is also of interest to know whether substitution of the thiourea molecule affects its stimulating nature. This also might assist in elucidating the mechanism of its action.

METHODS

Throughout the experiments lettuce seeds variety Grand Rapids, kindly provided by Messrs. Ferry Morse,¹ were used. The seeds were germinated as described in previous papers (Mayer and Evenari, 1952). Thiourea was estimated by the methods of Chesley (1944). This method is based on the development of a blue colour of the thiourea with derivatives of nitroferricyanide (Grote's reagent) and found to be more satisfactory than other methods described for the determination of thiourea (Danowsky, 1944; Campbell, 1944).

When thiourea was determined in the seeds, the following procedure was adopted. The seeds were washed with distilled water and ground in a mortar. They were then transferred to centrifuge tube and the proteins precipitated with tungstic acid (Chesley, 1944). The whole was then centrifuged and the supernatant used for thiourea determination.

¹ Our thanks are due to Messrs. Ferry Morse Seed Co., Mountain View, California.

RESULTS

Quantitative Determinations. The change in concentration of the external medium in which the seeds were germinated was studied after 24 and 48 hours. As will be seen from Table I, there was a 1 per cent. change of concentration of the external solution after 24 hours, and 9.6 per cent. after 48 hours.

Balance sheets of the total change in thiourea concentration after 48 and 72 hours were constructed. 500 mg. of seeds were germinated for 48 or 72 hours, and the amount of thiourea originally added (as determined from a control without seeds), the amount remaining in the seeds and the amount in the external solution were determined. The data record a number of experiments, each of which is the mean of three determinations. As will be seen from Table II there is no gain or loss in the total amount of thiourea, such changes as are observed being well within the experimental error of the method.

The amount of thiourea entering the seeds was found to vary with its external concentration, as is to be expected from the finding that thiourea enters together with the water (Table III).

It is also worth noting that the amount of thiourea absorbed increases markedly with time, cf. Table II.

The Effect of Substitution on the Stimulatory Action of Thiourea. Thiourea is remarkable for the fact that it stimulates germination within a very wide concentration range. Its concentration can be increased ten-fold without any inhibition being noticeable (Table IV).

In contrast to this, a number of thiourea derivatives which were tested all strongly inhibited germination in the concentration range in which thiourea stimulates. No evidence for a stimulating effect could be observed for any of these. The thiourea derivatives tested (*p*-tolylthiourea, *o*-tolylthiourea, *m*-tolylthiourea, R-benzylisothiurea hydrochloride, S-methylisothiurea sulphate, *iso*-thiureidopropionic acid, allylthiourea, phenylthiourea, 1:3-diethylthiourea) all cause inhibition in the concentration range of 0.35 to 1.6×10^{-2} M. In this range thiourea causes stimulation of germination. Substitution anywhere in the molecule of thiourea destroys its activity as a germination stimulator.

DISCUSSION

It is obvious that thiourea enters the seeds at almost the same rate at which water enters, particularly during the first 24 hours of germination.

Since thiourea enters the seeds in larger amounts after 72 hours than after 48 hours, it may be assumed that the amount entering during the first 24 hours is considerably smaller (Tables III and IV). This is also borne out by the changes in the concentrations of the external solution (Tables I and II).

It is, therefore, an obvious possibility that the small amount entering during the first 24 hours which is approximately the amount unaccounted for, is responsible for germination, particularly as the root emergence begins normally around the 18th hour.

It is also clear that the bulk of the thiourea entering after 24 hours is not

TABLE I

Change of Thiourea Concentration after Various Periods of Germination in Thiourea Concentration 1.25 mg./ml.

No. of trials	Time of germination (hours)	Range of percentage germination	Mean change in concentration mg./ml
5	24	14-79	-0.016 (-1.3%)
4	48	69-93	-0.12 (-9.6%)

TABLE II

Balance Sheet for Thiourea after 48 and 72 hours of Germination, Original Amount in each case 7.5 mg.

	Remaining outside seed	Found in seed	Loss or gain
48 hours	5.2	0.93	-1.37
	7.3	0.74	+0.54
	7.1	0.85	+0.45
	7.0	0.55	+0.02
72 hours	3.3	4.3	+0.1
	4.12	3.2	-0.18

Average amount unaccounted for after 48 hours of germination = 0.09 mg. thiourea/500 mg. seeds

Average amount unaccounted for after 72 hours of germination = 0.04 mg. thiourea/500 mg. seeds

TABLE III

Relation between External Thiourea Concentration and its Recovery from the Seeds

Total amount of thiourea provided outside mg.	Thiourea recovered from 500 mg. seeds after 48 hrs. mg.
1.25	0.12
2.5	0.18
5.0	0.44
7.5	0.61

TABLE IV

Variation in Germination Percentage of Seeds Germinated in Various Concentrations of Thiourea during 48 hrs.

Thiourea concentration $\times 10^{-2}$ M	Percentage germination
0	49
0.14	73
0.27	81
0.55	80
1.11	90
1.66	93

metabolized, and if bound, is bound so loosely that mere water extraction frees it. This bulk evidently does not combine permanently with any vital cell component. It is also evident that the molecule of thiourea must be unsubstituted for it to be effective as a stimulator. Both the sulphur and the two amino groups must apparently be free, which suggests that there is some form of attachment of the molecule at three points to a substrate. It is also possible that thiourea acts in some way as an enzyme activator. The effect of substitution of thiourea would then be merely steric. The molecule increases in size, and therefore cannot reach its point of action or having reached it cannot act. This still fails to explain, however, the strong inhibitive effect of thiourea derivatives.

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A Study of Nitrate Reduction in Mould Fungi

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Received 15 June 1955

SUMMARY

1. The activity of the nitrate-reducing (nitratase) system in fungal mycelium was measured by incubation of the mycelium (either intact or ground) with nitrate in the presence of sodium fluoride which blocks nitrite reduction and causes nitrite accumulation.

2. The nitrate-reducing system in the fungi examined shows rapid changes in activity in response to some environmental factors. Nitratase activity of mycelium in glucose-nitrate medium falls to a low value within 1 hour after addition of ammonia and remains at this value until all added ammonia has been assimilated. The fall in activity appears to depend on the assimilation of ammonia by the mycelium, since it did not occur in conditions when assimilation was prevented.

3. Nitrate-reducing activity declines rapidly in the absence of assimilable carbohydrate, and recovers equally rapidly when carbohydrate is restored. Recovery is accelerated by nitrate but almost completely prevented by ammonia.

4. Evidence is given that these variations in nitrate reduction are primarily changes in the nitrate reductase link in the enzyme system. Nutritional behaviour of the fungi investigated is closely correlated with the observed changes in activity of their nitrate reductase.

5. Nitrate reductase may be formed by these fungi when grown in complete absence of nitrate.

INTRODUCTION

IN a number of mould fungi the assimilation of ammonia (ammonium ion) by the fungus has been found to prevent the simultaneous assimilation of nitrate but not of nitrite (Morton and MacMillan, 1954). The presence of ammonia appeared to inhibit the reduction of nitrate to nitrite. These observations led to a study of nitrate-reducing activity in these fungi.

The experiments were begun when the nature of the nitrate-reducing system was still rather obscure. Recently the work of Nason and Evans (1953), Nicholas, Nason and McElroy (1954), Nicholas and Nason (1954) with fungi, and of Evans and Nason (1953) with higher plants, has greatly clarified the nature of the nitratase system. The experiments reported in this paper were not directed to the isolation of the enzyme components of the nitratase system, but to a study of the activity of the intact system. All the results obtained, however, were consistent with the presence in the fungi investigated of a nitrate reductase linked to triphosphopyridinenucleotide (TPN), as found by Nason and Evans in *Neurospora* and *Aspergillus*.

The term nitrate reductase will be used to designate the enzymatic link between nitrate and the immediate electron-carrier (reduced TPN) whilst the term nitratase system will be used in a wider sense to include both nitrate

reductase and those dehydrogenases and their substrates which are linked to nitrate reductase through TPN.

MATERIAL

The main experimental material was young actively growing mycelium of *Scopulariopsis brevicaulis* (Sacc.) Bainier. In addition *Penicillium griseofulvum* Dierckx, *Myrothecium verrucaria* (Alb. and Schw.) Ditm., and a mutant form of *Aspergillus nidulans* (Eidam) Winter, were used.

The fungi were grown at 25°, both in submerged (shaken) and in surface culture, in a standard nitrate culture medium with the addition of essential trace elements, including molybdenum. Details of culture medium and methods will be found in previous papers (Morton and MacMillan, 1954; Morton and Broadbent, 1955). Cultural conditions were thus favourable for the development of the nitratae system. Only young, actively assimilating mycelium (2 to 4 days after inoculation) was used for experiment.

THE MEASUREMENT OF NITRATE-REDUCING ACTIVITY

(1) *Intact mycelium.* It was found that the activity of the nitrate-reducing system could be demonstrated by incubating intact mycelium in phosphate buffer containing potassium nitrate in presence of 0.2 M. sodium fluoride. In these conditions the nitratae system continues to function for some hours, and nitrite accumulates. The presence of fluoride is essential: only traces of nitrite are formed in the medium in the absence of fluoride, even when the mycelium is incubated in an atmosphere of nitrogen, since nitrite is further reduced about as rapidly as it is formed. When fluoride is added the accumulation of nitrite proceeds at almost the same rate whether the mycelium is incubated in air or nitrogen.

The primary effect of sodium fluoride was shown to be to inhibit completely the metabolism of nitrite by the mycelium, whilst leaving the nitrate-reducing system less affected. The accumulation of nitrite in the presence of 0.2 M. sodium fluoride (lower concentrations were less effective) is thus a measure of the activity of the nitrate-reducing system at any time. The rate of nitrate-reduction in the first 3 to 4 hours incubation with fluoride (at 25°) was usually 0.2–0.4 mg. N/g. dry matter per hour which may be compared with a rate of about 1.5 mg. N/g. dry matter per hour for growing mycelium in favourable physiological conditions and at the same temperature. Intact mycelium in the test conditions may continue to form nitrite for over 20 hours but the rate is lower after the first 3 to 4 hours.

Whilst the main effect of fluoride is to prevent metabolism of nitrite by the mycelium, it has at least two further effects of importance in relation to the demonstration of nitratae activity. In the first place fluoride largely blocks the oxidative processes connected with the cytochrome system (Borei, 1945) and thus stops the diversion to atmospheric oxygen of the bulk of the hydrogen derived from metabolites in the cell. This effect of fluoride is shown by the fact that in presence of air intact mycelium will not appreciably reduce

triphenyltetrazolium chloride, energetic reduction, however, takes place, when 0.2 M. sodium fluoride is added. Secondly, fluoride was found markedly to retard the non-enzymatic reoxidation of nitrite by atmospheric oxygen both in pure solutions and in contact with heat-killed mycelium. Tests for nitratase activity were normally carried out in an atmosphere of nitrogen to minimize loss of nitrite by oxidation. Parallel tests with access of air showed only slightly lower results, however, owing to this protective action of fluoride. There was no evidence of a direct action of oxygen on nitratase activity.

The continued formation of nitrite for several hours by mycelium incubated in the test conditions clearly depends on the presence in the cells of substrates functioning as electron donors linked to the reduction of nitrate. Mycelium was extracted with boiling water and the cooled filtered extract was added to intact mycelium incubated with nitrate and fluoride in the test conditions. The amount of nitrite formed was consistently 10 to 20 per cent. greater than in control tests without added extract, indicating the presence in mycelial extract of substrates serving as electron donors. The effect of mycelial extract will be discussed in more detail later.

These observations formed the basis of a method for comparing the nitrate-reducing activity of fungal mycelium grown in different experimental conditions. Mycelium was separated from the culture fluid by filtration on a Buchner filter but was not washed. Samples of known fresh weight (usually 0.1 g.) were then transferred to tubes containing 1 ml. 0.07 M. phosphate buffer (pH 6.9), 1 ml. saturated NaF (final concentration 0.2 M.), 1 ml. 0.2 per cent. KNO_3 , 1 ml. water (replaced by hot-water extract of mycelium or other substrates, if desired). Air in the tubes was replaced by nitrogen gas before adding the mycelium. The tubes were stoppered and incubated at 25° for a standard time of 3 hours. Nitrite was determined colorimetrically after addition of the *a*-naphthylamine reagent of Parker (1949). Since soluble fluoride interferes, 1 ml. of a 20 per cent. (w/v) solution of hydrated CaCl_2 in ethanol was added before adding the Parker reagent (5 ml.). After full development of the colour (30 minutes) the solution was filtered and the nitrite was determined in a visual colorimeter by comparison with a nitrite standard freshly made up in the test solution and treated in exactly the same way. The amount of nitrite formed in the standard period was taken as a measure of the nitrate-reducing activity of the mycelium at the time it was harvested.

(2) *Ground mycelium.* An alternative method of testing the nitrate-reducing activity of mycelium was used, in which the cell-structure was first disrupted by freezing and grinding. Mycelium was harvested by filtration, and a known weight was quickly frozen by means of solid CO_2 and was then ground to a fine suspension in the same test solution as was used for intact mycelium (phosphate buffer with 0.2 per cent. KNO_3 and 0.2 M. NaF). The mycelial suspension was then incubated for a standard period (1 hour) at 25° in an atmosphere of nitrogen gas. The nitrite formed was determined as already described in the test with intact mycelium, and was taken as a comparative measure of nitratase activity.

During the first 1 to 2 hours nitrite is formed at about the same rate as when an intact sample of the same mycelium is incubated with fluoride and nitrate in the test conditions. The ground system is, however, more sensitive to the addition of electron donors than is intact mycelium, possibly because the disruption of cell structure allows freer access of substrates to the enzyme centres, and the amount of nitrite formed may be more than doubled by adding hot-water extract of mycelium. The ground system is less stable than intact mycelium, however, and the rate of nitrate reduction falls rapidly after the first 1 to 2 hours to a low level which may then be maintained for some hours. This is due to the decay of some components of the enzyme system, for the addition of more hot-water mycelial extract at this stage did not appreciably increase nitrite formation.

Both methods of test were applied simultaneously in some experiments and were found to give essentially the same results for the relative nitrate-reducing activity of the same series of mycelial samples. Thus both methods appear to give at least a rough quantitative measure of the nitratase activity of fungal mycelium at the moment when it is harvested for test. Relative activities for a series of samples determined on one occasion would seem generally to have more significance than absolute values, which were found to be somewhat sensitive to variations in experimental procedure.

As with intact mycelium, the presence of a high concentration of fluoride was required with ground mycelium in order to show nitratase activity by the formation of nitrite. The action of fluoride is, however, more complex in relation to the ground system. It inhibits the oxygen-linked enzymes and also protects nitrite from (non-enzymic) reoxidation, as with intact mycelium. This was indicated by the fact that exclusion of oxygen during incubation made relatively little difference to the results when fluoride was present. The blocking of nitrite reduction by fluoride is of less significance in ground than in intact mycelium, however, since the nitrite-reducing activity was usually negligible in ground preparations.

Sodium fluoride has in addition certain effects on the ground system which are not shown by intact mycelium. In the first place the inactivation of the nitratase system, which always occurs more or less rapidly in ground mycelium, was shown to be delayed by the presence of fluoride. Secondly, fluoride has the effect of preventing or retarding the loss from ground mycelium of the endogenous electron donors linked with nitrate reduction, as is indicated by the following observations. When frozen mycelium is ground in presence of nitrate and fluoride (test conditions) nitrite is formed at the expense of electron donors present in the ground material. When, however, frozen mycelium is ground in buffer alone and allowed to stand for 15 to 30 minutes before adding sodium fluoride and potassium nitrate, very little or no nitrite is formed on subsequent incubation unless hot-water extract of mycelium is added. Evidently the substrates linked to nitrate reduction are rather rapidly removed by some reaction which proceeds in ground mycelium, and which is strongly inhibited by sodium fluoride. The nature of this reaction

is not known. The reaction appears to be enzymic (since the electron donors are stable in heated extracts of mycelium) and has been shown to occur in the complete absence of oxygen.

ENDOGENOUS ELECTRON DONORS

As already mentioned the addition of a hot-water mycelial extract to ground or intact mycelium incubated with nitrate in presence of 0.2 M. sodium fluoride leads to an increase in the amount of nitrite formed. This suggested the presence of substrates (or possibly co-factors) in the extract which were linked as electron donors to nitrate reduction. Hot-water extract of mycelium was therefore treated with charcoal, which is known to adsorb the nucleotide coenzymes in the conditions employed (Le Page and Mueller, 1949). The extract after removal of the charcoal was only 10 per cent. less active than before in stimulating nitrite formation in a ground mycelium test system. The charcoal was treated with pyridine (procedure of Le Page and Mueller) to elute the coenzymes and this fraction was also tested with the ground mycelium system. It showed a very slight stimulating effect on nitrite formation, not significantly enhanced in the presence of the charcoal-treated extract, which suggests that the main effect of hot-water mycelial extract is due to the presence of electron donors.

The addition to a ground mycelium test system of a large number of substances dehydrogenated by DPN-linked enzymes was without effect on nitrite formation. A marked increase in nitrite formation was caused by adding glucose-6-phosphate, which is dehydrogenated by a TPN-linked enzyme, but *isocitrate* and glutamate (also TPN-linked) only showed just perceptible effects. These results show that the nitrate reductase in *Scopulariopsis* is linked to reduced TPN as in *Neurospora* and other fungi.

The main effective electron donor in the mycelium does not, however, appear to be glucose-6-phosphate since paper chromatography of the mycelial extract did not reveal recognizable amounts of glucose-6-phosphate. The effective substrate in mycelial extract was also very unstable to acid hydrolysis, whilst glucose-6-phosphate was shown to be stable in the same conditions. The precise nature of the effective electron donors in the mycelium is still under investigation.

NITRATASE ACTIVITY AND AMMONIA

The effect of ammonium ion on nitratase activity was studied in the following way. *Scopulariopsis brevicaulis* was grown in submerged culture on a reciprocating shaker in a complete nitrate medium, and at 2 to 3 days, when growth and nitrate assimilation were active, the culture fluid with suspended mycelium was divided into two parts. One part was regarded as a control and received no addition. To the other part was added sufficient ammonium tartrate, dissolved in the minimal amount of water, to make the concentration of ammonia equal to that of nitrate in the original medium (0.02 M.). The actual concentration of nitrate in the culture fluid at this stage varied but was

usually 50 to 80 per cent. of the initial concentration. The tartrate was used in order to obviate marked changes in pH of the culture fluid due to ammonia assimilation. Samples of mycelium were taken from both the control and experimental flasks immediately after the addition of ammonium tartrate to the latter, and tested for nitratability (intact mycelium).

The flasks were placed on the shaker and further samples of mycelium were removed at intervals and likewise tested for nitratability. The results of several typical experiments are shown in Fig. 1 where the nitratability

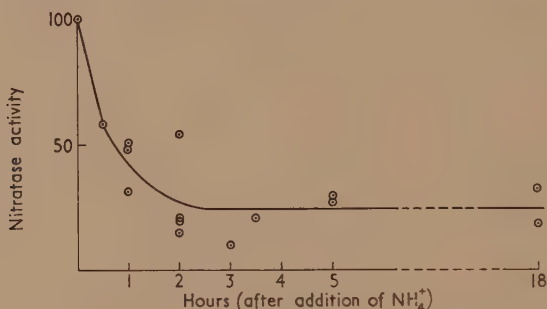


FIG. 1. Nitratability activities of *Scopulariopsis brevicaulis* as percentage of that of mycelium in nitrate shown as ordinates. Times after addition of 0.02 M. ammonium tartrate to the culture medium shown as abscissae.

activity of mycelium at various times after the addition of ammonium tartrate is given as percentage of the nitratability activity of the corresponding control mycelium in nitrate. It will be seen that the addition of ammonium ion to mycelium growing in nitrate medium causes a rapid fall in the activity of the nitrate-reducing system within 1 to 2 hours to a value which is only 20 to 30 per cent. of that in nitrate alone. The nitratability activity of the control mycelium in nitrate showed no consistent change with time over periods of 5 to 6 hours, although the actual values sometimes fluctuated as much as ± 30 per cent. between occasions. This variation between occasions could not be eliminated and seemed to be associated with slight but unavoidable differences in the treatment of mycelium (filtering, degree of removal of culture fluid, time of exposure to air) between harvesting and placing in the test conditions. Duplicate determinations of nitratability activity of one batch of mycelium on a single occasion always agreed more closely (usually ± 5 per cent.).

The reduction of nitratability activity of nitrate-grown mycelium by the addition of ammonium ion has been observed repeatedly with mycelium of different ages grown both in surface and in submerged culture. The data collected in Table I refer to *Scopulariopsis brevicaulis* and *Penicillium griseofulvum*: essentially similar observations were made with *Myrothecium verrucaria*. No difference in behaviour was observed between surface and submerged cultures. Whilst the magnitude of the effect of ammonia showed considerable variation, nitratability activity was usually reduced to 20 to 30 per cent. of that in nitrate alone. Since nitrate alone is normally assimilated by *Scopulariopsis brevicaulis*

at less than half the rate at which ammonia is assimilated (Morton and MacMillan, 1954), this means that in the presence of ammonia the assimilation of nitrate would be reduced to 10 to 15 per cent. of the rate of ammonia uptake. This corresponds closely to what was observed. The rate of loss of nitratase activity and the level attained were not significantly affected by the concentration of added ammonia over the range 0.02 to 0.002 M.

TABLE I

The effect of the addition of ammonium ion on the nitratase activity of living mycelium growing in nitrate medium

Culture	Age (days)	Nitratase activity 3 hours after adding NH_4^+ (as % of control in NO_3^- alone: intact mycelium test)	Range	No. of experiments
<i>Scopulariopsis brevicaulis</i>				
Surface	3-7	28.4 ± 21.2	0-62	12
Submerged (shaken)	2-4	24.9 ± 15.2	0-54	11
<i>Penicillium griseofulvum</i>				
Submerged (shaken)	2-3	15.0 ± 14.9	0-35	5

In some of the experiments, the rate of nitrite reduction by the mycelium was also determined, by incubating samples with nitrite. It was found that the rate of nitrite reduction by mycelium was not lowered by incubation with ammonium tartrate, in agreement with earlier results on nitrogen uptake to which reference has been made.

The effect of ammonia on nitratase activity was also tested on a mutant of *Aspergillus nidulans*, kindly supplied by Dr. G. Pontecorvo. This mutant grew on ammonia but not on nitrate or nitrite as the nitrogen source. Investigation in our laboratory showed that the nitratase system was intact but that the reduction (and assimilation) of nitrite was blocked. When the living mycelium was placed in a medium containing nitrate it did not grow, but nitrate was in fact reduced for many hours at a steady rate equal to the rate in *Scopulariopsis brevicaulis* growing in physiological conditions, whilst nitrite accumulated in the medium. This mutant was particularly valuable experimental material, since the nitratase activity could be readily determined in completely physiological conditions without the addition of a high concentration of sodium fluoride. The fungus was grown in submerged culture with casein hydrolysate as the nitrogen source, a little nitrate being added to the medium to ensure development of the nitratase system. Two day old mycelium was removed from the original culture medium, which at this stage gave a strong reaction when tested for nitrite, showing that the nitrate-reducing system had been formed. The mycelium was divided into two parts. One part was put in complete culture medium containing potassium

nitrate as the sole source of nitrogen. The other part was put into the same medium containing (in addition to potassium nitrate) ammonium tartrate of equivalent nitrogen content. Both lots of mycelium were then incubated with shaking in the normal conditions of submerged culture and the rate of formation of nitrite was observed. The results are shown in Fig. 2, from

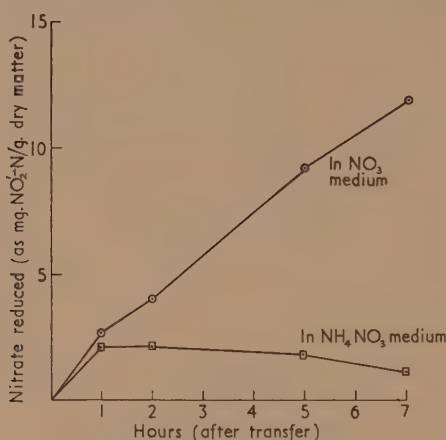


FIG. 2. Nitrate reduction of *Aspergillus nidulans* (NO₃ blocked mutant) in presence and absence of NH₄⁺.

which it is seen that the effect of adding ammonia is to reduce nitrataase activity to zero in about 1 hour. This result is important not only in confirming the effect of ammonia on nitrataase activity, but also as showing that tests with normal mycelium in the presence of fluoride give a real indication of nitrataase activity.

An inactivating effect on nitrataase activity is not shown by amino-acids in the presence of nitrate. The data in Table II show that there was no significant reduction of nitrataase activity when casein hydrolysate (amino-nitrogen equivalent to usual addition of ammonia-nitrogen) was added to mycelium growing in submerged culture in nitrate medium.

TABLE II

Nitrataase activity of mycelium in nitrate medium 2 hours after adding ammonium tartrate or amino acids

	Nitrataase activity (as % mycelium in nitrate only)—intact mycelium test		
	NO ₃ ⁻	NO ₃ ⁻ + NH ₄ ⁺	NO ₃ ⁻ + amino-acids
<i>Scopulariopsis brevicaulis</i>	100	20	97
<i>Penicillium griseofulvum</i>	100	22	98

The effect of ammonia in lowering nitrataase activity appears to be connected with actual assimilation of ammonia by the fungus. This view is supported by the following experimental evidence.

1. The addition of ammonium tartrate to the test system (i.e. to mycelium whether intact or ground, already in 0.2 M. sodium fluoride) had no effect on the rate of nitrite formation. Thus ammonia does not act as a direct inhibitor of nitrataase activity. It only inhibits if the mycelium has already been incubated with ammonia for some time in physiological conditions. True inhibitors of nitrataase activity such as cyanide, azide or thiourea suppress activity immediately and completely when added to the test system.

2. The addition of these inhibitors to intact growing mycelium also caused the immediate and complete loss of nitrataase activity. The addition of ammonium tartrate, however, did not cause the complete loss of nitrataase activity, although activity was sometimes as much as 20 per cent. lower within 2 to 4 minutes after addition, which is in practice the earliest observation that can be made. Usually the change in nitrataase activity immediately after addition of ammonium tartrate was not appreciable. The fall in nitrataase activity to a low level always took about 1 hour in the given experimental conditions, which suggests that it is a response to the assimilation of ammonia.

TABLE III

Effect of ammonia on nitrataase activity of Scopulariopsis brevicaulis in the absence of oxygen

Nitrataase activity following the addition of ammonium tartrate (as % activity of mycelium in nitrate in air: intact mycelium test)					
Expt.	Time after addition of NH_4^+ (hours)	Air		Nitrogen	
		NO_3^-	$\text{NO}_3^- + \text{NH}_4^+$	NO_3^-	$\text{NO}_3^- + \text{NH}_4^+$
1	2	100	25	50	72
2	4	100	20	55	45
3	5	100	—	44	40

3. When oxygen was excluded from the experimental flasks (by passing in nitrogen gas), the differential effect of ammonia addition on nitrataase activity was abolished. In the absence of oxygen, nitrataase activity slowly declined, both in nitrate and in nitrate+ammonia but at practically the same rate (Table III). In air, on the other hand, the addition of ammonia always caused a rapid fall in nitrataase activity of the mycelium relative to that of mycelium in nitrate without addition. The absence of oxygen has previously been shown to prevent completely the assimilation of ammonia or nitrate by the fungus (Morton and MacMillan, 1954). Iodoacetate at 5×10^{-3} is known to inhibit ammonia uptake by *Scopulariopsis* but not nitrataase activity. When ammonia was added to mycelium in nitrate together with 5×10^{-3} M. iodoacetate the usual rapid decline in nitrataase activity was replaced by a slow steady fall. Thus in one experiment nitrataase activity fell to 13 per cent. of the control 2 hours after adding ammonia, but when ammonia and iodoacetate were added nitrataase activity only fell to 57 per cent. in the same period. Loss of nitrataase activity in ammonia was similarly prevented by 2:4-dinitrophenol (10^{-3} M.).

ANALYSIS OF THE EFFECT OF AMMONIA ON NITRATASE

The experiments described show that the presence of ammonia in the culture medium causes a rapid fall in nitratase activity of *Scopulariopsis brevicaulis* and other fungi to a low value which is maintained as long as ammonia is present. The effect of ammonia appears to depend on its assimilation by the fungus. It thus becomes of interest to know what part of the nitratase system is affected.

The effect of ammonia assimilation might be to reduce the rate at which hydrogen becomes available for nitrate reduction, by lowering the concentration of the hydrogen donors or even of the hydrogen carriers (coenzymes) in the mycelium. This possibility was tested in the following way. Ammonium tartrate was added to shake cultures of *Scopulariopsis brevicaulis* growing in nitrate medium in the conditions already described. Mycelial samples were taken from control (NO_3^-) and experimental ($\text{NO}_3^- + \text{NH}_4^+$) flasks 2 hours after the ammonia addition, and were tested for nitratase activity (ground mycelium). Nitratase activity was determined both with and without the addition of a hot-water extract from nitrate-grown mycelium. The addition of 1 ml. of such an extract is equivalent to adding 2 to 3 times the concentration of hydrogen donors (and coenzymes) normally present in the weight of nitrate-grown mycelium taken for test. The results of several experiments are given in Table IV. It is clear that the depression of nitratase activity

TABLE IV

Effect of mycelial extract (natural hydrogen-donors) on nitratase activity of nitrate-grown and ammonia-grown mycelium

Fungus	Period of growth in presence NH_4^+ (hours)	Nitratase activity (as % activity of mycelium in nitrate: ground mycelium test)			
		In NO_3^-		In $\text{NO}_3^- + \text{NH}_4^+$	
		No addition	+mycelial extract	No addition	+mycelial extract
<i>Scopulariopsis brevicaulis</i>	2	100	209	14	28
	2	100	115	38	62
	3	100	166	24	48
	72*	100	150	36	48
<i>Penicillium griseofulvum</i>	2	100	130	30	48

* In this case mycelium grown from inoculation in ammonium nitrate culture medium was compared with mycelium grown in nitrate medium.

caused by ammonia is not removed by the addition of hydrogen donors and coenzymes from fully active mycelium. The lower activity of the nitratase system in ammonia-treated mycelium thus results primarily from a change in catalytic activity of some enzyme component of the system, rather than from a change in substrate concentration. This has been confirmed in another way.

In several experiments equal fresh weights of mycelium were taken from 'nitrate' and 'ammonia' flasks at the stage ($1\frac{1}{2}$ to 2 hours after ammonia addition) when nitratase activity in the latter had fallen to a low value, and the mycelium was extracted with hot water. The concentration of hydrogen donors in the extracts was then compared by adding them to duplicate nitratase test systems consisting of ground mycelium in the presence of fluoride and nitrate. The increase in nitrate formation in a standard time (1 hour) above that in the control system without addition of hot-water extract was taken as the relative measure of the concentration of hydrogen donors in each extract. The results showed that the concentration of hydrogen donors in 'ammonia' mycelium was 80 per cent. (70 to 106 per cent.) of that in the corresponding 'nitrate' mycelium. The assimilation of ammonia appears therefore to cause a slight although not very consistent fall in the level of the hydrogen donors linked with nitrate reduction, but the loss of nitratase activity is much greater than can be accounted for by this change in substrate concentration. The low activity of nitratase in 'ammonia' mycelium seems unlikely to be due to lack of coenzymes or other cofactors, since the extract of 'nitrate' mycelium presumably contains such factors in addition to hydrogen donors.

The fall in nitratase activity induced by ammonia seems therefore to be due to a loss of enzyme activity affecting either the dehydrogenases linking the hydrogen donors with TPN, or nitrate reductase linking reduced TPN and nitrate, or perhaps both steps equally. It is possible to make an independent estimate of dehydrogenase activity by linking the system to 2:6-dichlorophenolindophenol and other oxidation-reduction indicators and measuring the time of decolorization of the dye. A nitrate-grown shaken culture of *Scopulariopsis brevicaulis* (3 days old) was divided into two parts and ammonium tartrate was added to one part; both lots of mycelium were then returned to the shaker. Samples of mycelium were taken for test initially (immediately after ammonia addition) and after 1.5 and 3 hours. Nitratase tests (intact mycelium) showed no loss of activity on adding the ammonium tartrate but after 1.5 and 3 hours activity in the 'ammonia' mycelium had fallen to 10 to 15 per cent. of that in the 'nitrate' mycelium. For dehydrogenase tests samples of mycelium (0.5 g. fresh weight) were suspended in 2 per cent. glucose in phosphate buffer (pH 6.9) in stoppered tubes kept at 25°. Air was removed by a stream of nitrogen, 2:6-dichlorophenolindophenol was added to make the final concentration 1.7×10^{-4} M., and the time for decolorization was noted. Duplicate tubes were set up with and without 0.2 M. sodium fluoride. The purpose of adding sodium fluoride to one set of tubes was to make the conditions of test the same as those normally used for nitratase tests. The results of two experiments are given in Table V. There is clearly no evidence of a reduction in dehydrogenase activity in the 'ammonia' mycelium although nitrite formation was sharply reduced. This points to an effect of ammonia on the activity of the nitrate reductase part of the system. It will be noted that the rate of reduction of the dye is consistently higher

in the presence of fluoride. The reason for this is not known but it possibly indicates a stabilizing effect of fluoride on the dehydrogenases (compare the stabilizing effect of fluoride on the nitratase system, mentioned earlier).

TABLE V

Dehydrogenase activity of Scopulariopsis brevicaulis (measured as time in minutes of decolorization of 2:6-dichlorophenolindophenol) in nitrate and ammonia

Mycelium in	Exp. 1		Exp. 2	
	NO_3^-	$\text{NO}_3^- + \text{NH}_4^+$	NO_3^-	$\text{NO}_3^- + \text{NH}_4^+$
Initially No addn.	14	14	23	22
+NaF	8	8	13½	14
1.5 hours No addn.	15	15	22	20
+NaF	10	10	10	11
3 hours No addn.	12	10	16	13
+NaF	8	5	7	9

In a further experiment of the same type, the dehydrogenase activity of *Penicillium griseofulvum* was determined by adding triphenyltetrazolium chloride to the ground mycelium in 0.2 M. sodium fluoride in phosphate buffer (pH 6.9) and measuring colorimetrically in ethanol solution the red formazan compound formed. Incubation of the mycelium with ammonium tartrate for 2 hours caused a fall of 70 per cent. in the nitratase activity in this experiment, but the dehydrogenase activity as measured by formazan production in the conditions described was only 10 per cent. lower than the control mycelium in nitrate. Both methods of measuring dehydrogenase activity thus suggest that the main effect of ammonia is to lower the activity of the nitrate reductase part of the nitratase system and not the dehydrogenase step. It should, however, be noted that the methods used give a measure of total dehydrogenase activity of the mycelium (both DPN-linked and TPN-linked) whilst only the TPN-linked activity is available for nitrate reduction.

NITRATASE ACTIVITY AND CARBOHYDRATE SUPPLY

The nitratase activity of mycelium has been found to depend on the presence of assimilable carbohydrate and to fall rapidly in its absence to a value too low to be detectable in the test conditions. Nitrate-grown mycelium of *Scopulariopsis brevicaulis*, initially with an active nitratase system, was shown to have lost all nitratase activity after being shaken for 2 to 3 hours in phosphate buffer alone (no glucose present) with access of air. This loss of nitratase activity in the absence of carbohydrate appears to be primarily a loss of nitrate reductase activity. The evidence for this is essentially the same as was adduced to show that ammonia primarily affects nitrate reductase. When nitratase activity had been lost by keeping mycelium without glucose the activity was not restored by adding mycelial extract rich in hydrogen donors. Furthermore the concentration of hydrogen donors was shown to be

not significantly reduced in mycelium shaken for 2 hours in buffer. Dehydrogenase activity (reduction of 2:6-dichlorophenolindophenol) was only 12 per cent. lower than initially, whereas nitratase activity was more than 90 per cent. lower.

Thus incubation of active mycelium without glucose causes practically complete loss of nitrate reductase activity in 2 to 3 hours. This rapid loss occurs in the presence of oxygen. In nitrogen there is also loss of nitratase activity but it is slower, and complete loss of activity takes 4 to 5 hours or even longer.

When glucose was once more supplied to mycelium previously shaken for 2 hours in buffer, nitrate reductase activity was fully recovered in the course of 2 to 3 hours, provided conditions were aerobic. No recovery of activity occurred with added glucose when oxygen was excluded, or, in aerobic conditions, in the presence of iodoacetate (5×10^{-3} M.) or 2:4-dinitrophenol (5×10^{-4} M.). Evidently the recovery of nitrate reductase activity depends on the assimilation of carbohydrate. This was confirmed by the fact that activity was also recovered in fructose or mannose, both readily assimilable by *Scopulariopsis brevicaulis*, but not in galactose or arabinose, which are only assimilated by the fungus after a long adaptive lag period.

The recovery of nitrate reductase activity by mycelium previously shaken without carbohydrate supply was also tested in the presence of different nitrogen sources. Recovery occurred in glucose+nitrate at the same rate as or somewhat faster than in glucose alone: there was no recovery in nitrate without the addition of glucose. Recovery occurred in glucose+nitrite in the same conditions. In contrast to this, the recovery of nitrate reductase activity in glucose was completely prevented during at least 3 hours by the presence of ammonium ion (as ammonium sulphate or ammonium tartrate) at 0.03 to 0.001 M. When present as ammonium nitrate there was usually a slight recovery of activity to a low level. These experiments thus confirm the effect of ammonia on nitratase activity which has already been described.

NITRATASE IN MYCELIUM GROWN ON AMMONIUM SALTS

The foregoing experiments have been concerned with mycelium grown in the first place on nitrate and with the effects of ammonium ion on the fully developed nitratase activity of such mycelium. It was therefore of interest to examine the nitratase activity of mycelium grown initially on media containing ammonia nitrogen. *Scopulariopsis brevicaulis* and *Penicillium griseofulvum* were grown in submerged shaken culture in the basic medium with nitrate, ammonium tartrate, and ammonium nitrate, as the respective sources of nitrogen.

Three-day old mycelium from each culture was tested for nitratase activity, using the ground mycelial system, immediately after removal from the medium. At the same time the 'nitrate' mycelium was transferred to fresh ammonium tartrate medium, whilst the 'ammonium tartrate' mycelium and the 'ammonium nitrate' mycelium were transferred to fresh nitrate medium. The

transferred mycelium was shaken for 1 hour in the new media and samples were then tested for nitratase activity. The results are shown in Table VI. It will be seen that the effects of ammonia on nitratase activity are essentially the same whether the fungi are grown on ammonia-nitrogen from the start or whether ammonia is added to mycelium growing on nitrate, although there are differences between the two species. When *Scopulariopsis brevicaulis* is grown on ammonia nitratase activity is at a low but definite level, slightly higher if nitrate is also present. In *Penicillium griseofulvum*, however, nitratase activity is not demonstrable in ammonia-grown mycelium even when nitratase is present.

TABLE VI

Nitratase activity of mycelium from different nitrogen sources (as per cent. activity of S. brevicaulis on nitrate: ground mycelium test)

Fungus	Grown (3 days) on	Nitratase activity	Transferred (1 hour) to	Nitratase activity
<i>Scopulariopsis brevicaulis</i>	NO_3^-	100	NH_4^+	10
	NH_4^+	16	NO_3^-	24
	$\text{NH}_4^+ + \text{NO}_3^-$	32	NO_3^-	46
<i>Penicillium griseofulvum</i>	NO_3^-	12	NH_4^+	0
	NH_4^+	0	NO_3^-	29
	$\text{NH}_4^+ + \text{NO}_3^-$	0	NO_3^-	32

The transfer experiments show that nitratase activity develops very rapidly, once ammonia is removed. The rate of development is of the same order as the rate at which activity is lost after the addition of ammonia. In another experiment ammonia-grown mycelium of *Penicillium griseofulvum* with no demonstrable nitratase activity was transferred to glucose-nitrate medium and to the same medium without nitrate. In glucose-nitrate nitratase activity was fully developed in 1 hour and maintained almost the same level at 3 hours. In glucose without nitrate nitratase activity was just detectable in 1 hour and by 3 hours had risen to 36 per cent. of the activity of mycelium in glucose with nitrate. It is thus clear that nitrate reductase is formed by the fungus in complete absence of nitrate (so long as ammonia is also absent), although nitrate increases the rate of formation.

DISCUSSION

The most interesting conclusion arising from the results is the extreme lability of the nitratase system in these fungi. The activity of the system mediating reduction of nitrate to nitrite has been shown to vary rapidly and widely in response to physiological changes in the cell depending on external factors. Whilst these changes probably have effects on other parts of the nitratase system, the evidence suggests that the most labile part is actually nitrate reductase, the catalytic link between nitrate and reduced TPN. Recently Silver and McElroy (1954) observed the rapid development of

nitrate reductase in *Neurospora*, a finding similar to the results in *Scopulariopsis* and *Penicillium*.

The activity of nitrate reductase depends on a supply of assimilable carbohydrate and falls rapidly to zero, or a very low value, in its absence. When mycelium is growing in physiological conditions, however, with adequate carbohydrate supply, the most marked effect is exercised by ammonium ion which causes a rapid fall in nitrate reductase activity to a low value which is maintained whilst ammonium ion is present. The effect does not seem to be related to concentration of the ammonium ion over a wide range. In these conditions the rate of assimilation of ammonium ion by the mycelium was also found to be independent of its concentration over a similar range (Morton and MacMillan, 1954). Evidence has been given which at least strongly supports the view that the fall in nitrate reductase activity in presence of ammonium ion is associated with ammonia assimilation by the fungus, since this effect does not occur when assimilation is prevented by absence of oxygen or by addition of iodoacetate. Mycelium growing in nitrate medium has been shown (loc. cit.) to be capable of assimilating ammonia at maximal rate without lag. When ammonia is added to such mycelium its assimilation therefore begins immediately, resulting in a rapid fall in nitrate reductase activity which contributes to the blocking of any further assimilation of nitrate as long as ammonia is present.

How the assimilation of ammonia affects nitrate reductase is not yet clear. The nitrate reductase link has been shown by Nicholas and Nason (1954) to be quite a complex system in itself. The effect of ammonia assimilation might be exercised on oxidation-reduction potential in the cell, or in other ways. Various possibilities are being examined. When nitrate reductase activity is lost through starvation or ammonia treatment, it is regained when the mycelium is placed in glucose in the absence of any nitrogen source. This perhaps indicates the passage of the enzyme from an inactive to an active form, rather than the complete resynthesis of an enzyme which has been broken down.

The changes in nitratase activity correspond to the physiological facts previously observed, and account for the blocking of nitrate, but not of nitrite, uptake in the presence of ammonium ion. Amino-acids in the form of casein hydrolysate do not lower the activity of nitrate reductase in presence of nitrate, and it has been found (unpublished) that nitrate and amino nitrogen are taken up simultaneously. It is not, of course, suggested that the ammonia-induced fall in nitrate reductase activity in these fungi is the sole cause of their failure to assimilate nitrate in presence of ammonia. The loss of nitrate reductase in these conditions, although highly significant, is rarely complete. The rapid inactivation of the nitrate-reducing enzyme seems, however, to be the primary phase, although it is doubtless followed by other metabolic adjustments leading to complete loss of nitrate uptake.

The blockage of nitrate uptake in presence of ammonia-nitrogen was considered by Morton and MacMillan (1954) to be of adaptive significance in

natural environments by permitting the fungi in question to utilize the available nitrogen most economically. Whilst many fungi show this type of relation between ammonia and nitrate nutrition, it should nevertheless be noted that there are others which do not. Unpublished observations of Dr. Anne MacMillan in this laboratory show that certain fungi which grow best in media with low pH values may assimilate nitrate and ammonia simultaneously, or even assimilate nitrate exclusively.

ACKNOWLEDGEMENTS

The author is indebted to Dr. G. Pontecorvo for kindly supplying the *Aspergillus* mutant. He wishes to express his gratitude to Mrs. V. J. Page and Mr. D. J. England for skilled technical assistance.

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The Entry of Ammonia into Fungal Cells

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Received 28 July 1954

SUMMARY

When *Scopulariopsis brevicaulis* grown on an ammonia-free medium is supplied with an ammonium salt ammonia enters the cells more rapidly than it is removed by assimilation, until an equilibrium level of ammonia is reached in the cells. The equilibrium concentration in the cells is independent of metabolism and depends on the external concentration over a wide range. The internal concentration of ammonia can be higher than the external under suitable conditions of pH. The cells are shown to be permeable to ammonia also in the outward direction, and the rate of entry or loss depends on the concentration difference between external and internal environment.

The results support the view that ammonia enters the cells mainly by the free diffusion of the undissociated molecule.

INTRODUCTION

THE presence of ammonia in the culture medium has a profound effect on the assimilation of other nitrogenous substrates by fungi. In particular nitrate assimilation is completely inhibited by the presence of ammonia (Morton and MacMillan, 1954). This has led to a study of the relationship between the assimilation of different forms of nitrogen and their mode of entry into the cell. The present paper deals with the entry of ammonia into fungal cells and the factors affecting its distribution between the cells and the surrounding culture fluid.

Relatively little is known specifically of the permeability of fungal cells, although many problems of fungal metabolism require an understanding of the ease with which solutes enter the mycelium. Ammonia, a weak electrolyte, is known to penetrate the cells of many organisms readily (Davson and Danielli, 1952) and the rate of entry and the equilibrium levels in the cell are thought to depend on the degree of dissociation as well as the permeability of the cell.

In these experiments suspensions of the mycelium were supplied with an ammonium salt under controlled conditions and changes in the ammonia content of the cells were determined in extracts of the mycelium and in some cases in the expressed cell sap.

The results have shown that ammonia equilibrates rapidly in the cells and under suitable conditions accumulates in the cells at a concentration many times greater than that of the external medium. The results in general are in agreement with the entry of ammonia in the form of the undissociated molecule.

Journal of Experimental Botany, Vol. 7, No. 19, pp. 113–26, February 1956.

The term ammonia is used in this paper in a general sense to include ammonia-nitrogen in the form of ions and undissociated molecules.

MATERIAL AND METHOD OF CULTURE

A strain of *Scopulariopsis brevicaulis* Sacc. (Bain.) has been used for most of this work. *Fusarium graminearum* Schwabe, *Aspergillus niger* v. Tieghem and *Penicillium griseofulvum* Dierckx were used in some of the experiments. Stock cultures were maintained on Czapek-Dox agar (nitrogen as nitrate).

The moulds were grown in a synthetic medium containing 5 per cent. glucose and nitrogen (33.5 mg. N/100 ml.) as potassium nitrate. The medium was dispensed in 250 ml. lots into 700 ml. flasks, inoculated with a spore inoculum, and shaken on a horizontal stroke shaker at 25° C. The moulds grew as evenly dispersed suspensions of mycelium. *S. brevicaulis* was harvested for experiment after 3 days growth, *P. griseofulvum*, *F. graminearum*, and *A. niger* after 2 (sometimes 1) days. The mycelium was filtered off and washed 2 or 3 times with distilled water before use.

EXPERIMENTAL METHODS

For most experiments the mycelium was then suspended in 0.07 M. orthophosphate buffer containing ammonia (as $(\text{NH}_4)_2\text{SO}_4$). The buffers contained pyrophosphate for pH values of 8 and above, and citric acid below pH 6. The mycelial suspension was shaken during the experimental period.

Determination of the uptake of ammonia. Samples of the suspension of known volume were withdrawn at intervals and filtered and the loss of ammonia from the solution per unit weight of mycelium was determined (Morton and MacMillan, 1954).

Extraction of mycelium for determination of ammonia content. A 1–3 g. sample of mycelium was dried in the mangle as described below, weighed quickly and heated in approx. 10 times its weight of phosphate buffer (pH 7) in a boiling water bath for 3 minutes. The extract was cooled and made up to volume, and ammonia determined on aliquots of the whole suspension and calculated as mg. N/g. fresh weight (F.Wt.). Added glutamine is hydrolysed less than 5 per cent. by this extraction procedure.

Fresh weight of mycelium. The mycelial suspension was filtered rapidly on a Buchner funnel at the water pump until no free liquid was visible. The mycelial pad was removed and passed 4 times through a small mangle between filter papers (6 on each side) before weighing. In this way fresh weight/dry weight ratios of replicate samples were reproducible, in the hands of one operator, to ± 3 per cent.

Pressing procedure for cell sap. The method of Crowdy and Pramer (1955) was used, the cells being killed by rapid treatment with ether and the sap expressed at about 350 lb. per sq. in. The cell sap was freed from ether by aeration before ammonia estimations were made.

Ammonia estimation in mycelial extracts and cell sap was done as described by Koch and Hanke (1948), the ammonia (10–100 μg . $\text{NH}_3\text{-N}$) being

liberated with borate buffer at pH 10.1. The distillate was collected for 5 minutes in dilute HCl and Nesslerized. Check estimations, using glutamine and asparagine, showed that they were not hydrolysed under these conditions.

In the solutions in which mycelium was shaken (which contained no amides) larger amounts of ammonia were estimated by the method of Conway and Byrne (1933), using boric acid as absorbent for ammonia. *pH* was determined with a 'Cambridge' bench pH meter.

Estimation of the liquid adhering to the mycelium. It is impossible to separate the mycelium completely from the surrounding solution by filtering or drying. If mycelium has been suspended in a solution containing ammonia then a high proportion of the ammonia in the extract made from it may have come from the solution adhering to the cells and wetting the cell walls. This adhering solution could be freed from ammonia by thorough washing of the mycelium before it is extracted, but it was found that ammonia leaks quite rapidly from the cells once they are transferred to an ammonia-free solution. Washing the cells would therefore introduce error into the determination of the ammonia in the cells.

To overcome this difficulty we adopted a procedure in which the ammonia present in the surface film is included in the mycelial extract, and a correction is made for it. An estimate was therefore required of the volume of extracellular liquid adhering to the mycelium under our conditions before it is extracted. This was made by the use of solutions of substances which do not readily enter the cells. The results of several experiments using glucose as the non-penetrating solute are shown in Table I. The mycelium was shaken for about 10 minutes in dilute glucose solutions in 0.07 M. phosphate buffer (pH 7). Control samples of mycelium were treated identically, except that no glucose was present in the buffer. Samples were then filtered and dried in the mangle as described, weighed rapidly, and transferred to 50 ml. portions of phosphate buffer, shaken for 1 minute and filtered. Glucose was determined in the filtrate and in the original solution by the anthrone method (Fairbairn, 1953). Glucose carried over on the mycelium into the glucose-free buffer solution in excess of that by the controls is assumed to have come from the adhering surface solution. The figures indicate that approximately 40 per cent. of the total fresh weight of our 'dried' mycelial samples represents adhering liquid. The reverse experiment in which a sample of mycelium was transferred from buffer to a known volume of the same buffer containing glucose (0.01 per cent. *w/v*) gave a similar value. Thus the dilution of the glucose solution due to the addition of mycelium gave a value of 0.42 ml. surface liquid per g. fresh weight.

Estimations using a solution of the negative stain, nigrosin (which was estimated colorimetrically) as the non-penetrating solute, gave an average value of 0.38 ml./g. fresh weight. The value of 0.40 ml./g. has therefore been taken as being a reasonable estimate of the adhering liquid in our experimental conditions.

Determination of internal ammonia concentration of mycelium. For calculation

of the 'internal' concentration of ammonia in the fungal cells we have assumed that

NH_3 found in mycelial extract/g. total F.Wt.

= NH_3 in 0.6 g. F.Wt. mycelium + NH_3 in 0.4 ml. external solution.

From this the NH_3 /g. F.Wt. (corrected) has been calculated throughout.

TABLE I

Determination of liquid adhering to mycelium. Measurement of glucose washed off mycelium which had been equilibrated with 0.2 per cent. w/v glucose solution (pH 7)

Expt. no.	Glucose in first soln. (mg./ml.)	Total wt. of mycelium (g.)	Glucose carried over into 50 ml. buffer soln. (mg.)	Calc. vol. of adhering soln. ml./g. of mycelium
109	9.3	5.17	22.5	0.46
	0	5.05	0.25	—
110	1.95	3.14	3.1	0.47
	0	2.94	0.15	—
111	1.81	4.64	3.78	0.42
	1.81	4.96	4.15	0.43
	0	4.95	0.25	—
112	0.94	6.26	2.0	0.31
	3.85	5.05	7.4	0.37
	3.85	5.40	8.0	0.38
	5.60	5.41	12.75	0.42
	0	4.85	0.2	—
			Average	0.402

RESULTS

Movement of ammonia into and out of the cells. When mycelium grown on ammonia-free medium was placed in buffer, pH 7, containing ammonium sulphate and glucose and shaken to ensure a supply of oxygen, there was a steady loss of ammonia from the solution continuing for several hours, showing that ammonia was being taken up by the mycelium (Fig. 1). Most of the ammonia taken up was assimilated into amino-acids and protein (MacMillan, 1952). Determination of free ammonia in the mycelium showed that this rose rapidly during the first few minutes and then more slowly, reaching a steady level of 0.4 mg. N/g. F.Wt. after 1 hour which was maintained for 6 hours. The rate of entry of ammonia initially is therefore faster than its removal by assimilation. During the first 1–2 minutes, that is by the time the first sample after transfer was taken, the rate of entry of ammonia is 6 mg. N/g. F.Wt./hr. After the steady equilibrium level of ammonia in the mycelium is reached, the rate of entry and therefore of assimilation is only 2 mg. N/g. F.Wt./hr.

If glucose is not supplied in the solution the rate of entry of ammonia follows an essentially similar course during the first hour. After 1 hour there is no

further uptake of ammonia, showing that assimilation has stopped. From these results it is evident that the level of ammonia in the cells is independent of the rate at which it is removed by assimilation. The initial rate of entry is faster than the rate of assimilation, and when assimilation stops, in the absence of glucose, the level of ammonia in the cells does not rise appreciably, although we have found that it is always slightly lower in the presence of glucose.

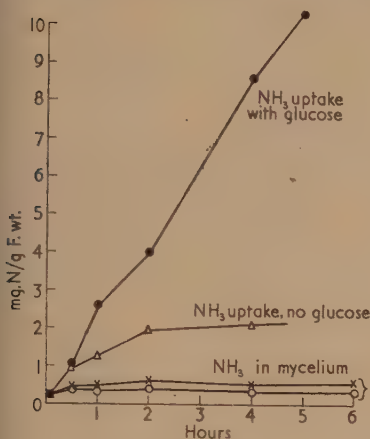


FIG. 1.

FIG. 1. The uptake of ammonia by *S. brevicaulis* from ammonium sulphate solution (pH 7, 0.4 mg. N/ml.) and the ammonia found in the mycelium (cf. Fig. 2). (—●—) NH_3 uptake 1% glucose, (—△—) NH_3 uptake no glucose, (—○—) NH_3 in mycelium 1% glucose, (—×—) NH_3 in mycelium no glucose.

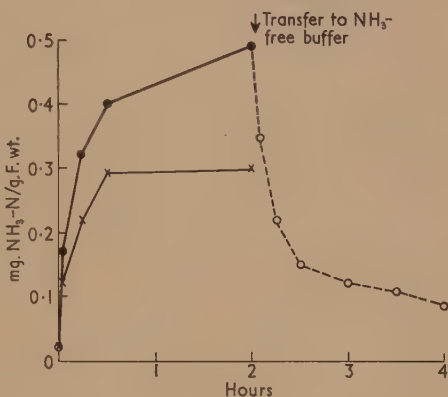


FIG. 2.

FIG. 2. Ammonia found in mycelium of *S. brevicaulis* shaken in phosphate buffer pH 7 containing $(\text{NH}_4)_2\text{SO}_4$ (0.4 mg. N/ml.). (—●—) No glucose, (—×—) 1% glucose, (---○---) transferred to ammonia-free buffer.

The rapid entry of ammonia into the cells is shown more clearly in Fig. 2 where the ammonia levels in the cells, obtained at more frequent intervals, are shown. It will be seen that ammonia is also lost rapidly from the mycelium when it is returned to ammonia-free buffer. Within 15 minutes the concentration of ammonia in the cells had fallen to below 50 per cent. of the equilibrium level of ammonia, after which it fell more slowly. Of the ammonia lost from the mycelium 90 to 95 per cent. was recovered from the washing solution. After 2 hours in this solution the ammonia level in the cells was still above the original so that it is possible that some is held more firmly, possibly by adsorption in or on the cells. The bulk of the ammonia present in the cells after shaking in ammonia solution is easily lost, however, and the cells are evidently readily permeable to ammonia in both directions.

The results suggest that ammonia enters by passive diffusion along the concentration gradient. Support for this comes from experiments with respiratory inhibitors. Preliminary experiments (Fig. 1) showed that ammonia entry is independent of an external energy supply. However, the endogenous respiration of this material is high and experiments in this laboratory have shown

that it is hardly increased by the addition of glucose. The entry of ammonia is therefore not necessarily independent of respiratory energy. However, if respiratory inhibitors are added at concentrations which inhibit endogenous respiration there is very little change in the rate and level of ammonia equilibrium (Table II). It is clear, therefore, that the level of ammonia in the cell is not dependent on a supply of respiratory energy; nor is it governed by the rate of removal of ammonia from the cell by assimilation, since the inhibitors used also inhibited ammonia assimilation almost completely.

TABLE II

The ammonia content of mycelium shaken 2-4 hours in $(\text{NH}_4)_2\text{SO}_4$ (0.4 mg. N/ml.) in phosphate buffer (pH 7) in the presence of respiratory inhibitors

Inhibitor	Molarity	Respiration rate as % of control	Nitrogen assim. as % of control	NH_3 Conc. in cells as % of control (aver.)
Iodoacetic acid	0.005	70	20	94
	0.01	65	—	100
2:4-dinitrophenol	0.00125	100	—	109
	0.0025	90	20	116
KCN	0.002	30	—	78
	0.005	30	< 30	115
NaN_3	0.01	60	30	102
	0.02	33	30	125

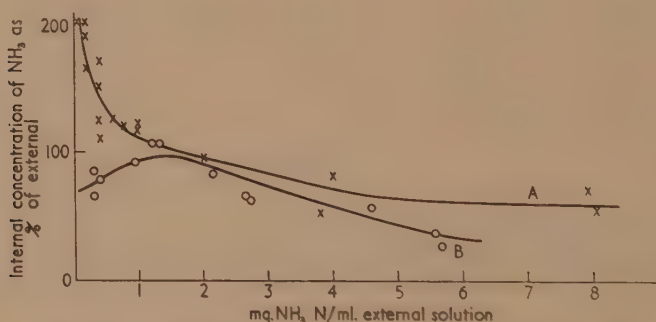


FIG. 3. Relation of internal to external ammonia concentrations. (A) Short term experiments. Mycelium shaken for 2-4 hours in phosphate buffer pH 7. (B) Culture experiments. Mycelium 4-6 days old growing in ammonia-media

We therefore investigated the part played by concentration differences in determining the accumulation of ammonia in the cells. The results of several experiments in which the mycelium was equilibrated with a range of external concentrations are shown in Fig. 3, curve A. Over a wide range, the internal concentration is about the same or slightly above the external. It is reasonable to conclude from these results that there is a passive equilibration of the internal ammonia with the external concentration. At the highest concentrations of ammonia (above 4 mg. N ml.) the internal concentrations fall below

the external. This may be due in part to insufficient time allowed for these high concentrations to be reached in the cells.

At the lowest external concentrations (below 0.05 mg. N/ml.) the internal concentrations are much higher than the external, again indicating that some adsorption on to parts of the cell may occur. At higher ammonia concentrations adsorption accounts for a smaller proportion of the total ammonia in the cells.

The relation between the external and internal ammonia levels holds not only under these short-term experimental conditions, but also when the fungus is growing in still culture (Fig. 3, curve B). Ammonium sulphate was used as nitrogen source and the medium buffered to pH 7.0–7.5 with calcium carbonate. With external concentrations up to 2 mg. NH_3 -N/ml. the concentration in the mycelium corresponds closely to the external. Thus under these conditions of active growth and ammonia assimilation, and with mycelium of various ages, we get the same picture of passive equilibration of internal with external concentration. At the higher concentrations of ammonia the internal concentration again falls below the external. There does not appear to be much adsorption at low external concentration under these conditions.

These estimations of 'internal concentration' have been made on extracts of the whole mycelium. Experiments in which the cell sap was expressed from cells equilibrated in ammonia solutions showed that the bulk of the ammonia in the cells is in solution in the cell sap, where its concentration corresponds roughly to the external. The liquid expressed from the mycelium consists of a mixture of external solution adhering to the cells and of cell sap. The ammonia concentration of the mixture, and therefore of the sap, is roughly the same as the external concentration over the range of concentrations used (Table III).

TABLE III

Ammonia found in expressed cell sap of S. brevicaulis after shaking 2 hours in $(\text{NH}_4)_2\text{SO}_4$ in phosphate buffer, pH 7. Cells ether treated

	External NH_3 concentration (mg. N/ml.)			
	0 (initial)	0.02	0.36	4.0
NH_3 in sap (mg. N/ml.)				
cells unwashed	0.013	0.026	0.40	3.5
cells washed before pressing	—	—	0.12	—
NH_3 in mycelium determined in boiled extract of mycelium (mg. N/g. F.Wt.)				
cells unwashed	0.02	0.03	0.39	3.2
cells washed before extracting	—	—	0.20	—

If the mycelium is first freed from external ammonia by rapid washing immediately before ether treatment, the expressed liquid still contains ammonia, which must have been in solution in the cells, but which is now diluted with ammonia-free surface liquid.

The rate of ammonia assimilation was found to be unaffected by ammonia concentration over a wide range (Table IV), so that differences in the rate of accumulation of ammonia in the mycelium indicate differences in the rate of entry. Fig. 4 shows that this rises with increasing external concentration. The differences in rate are most marked initially when the concentration differences

TABLE IV

Ammonia assimilated by mycelium of S. brevicaulis from solutions of ammonium sulphate in phosphate buffer (pH 7) in the presence of 1 per cent. dextrose

NH ₃ assimilated (mg./g. F.Wt. corrected)	Initial external NH ₃ concentration (mg. N/ml.)			
	0.047	0.091	0.47	0.90
1 hour	2.5	2.8	2.3	2.7
5 hours	9.2*	10.3	10.2	10.5

* Ammonia supply exhausted before end of experiment.

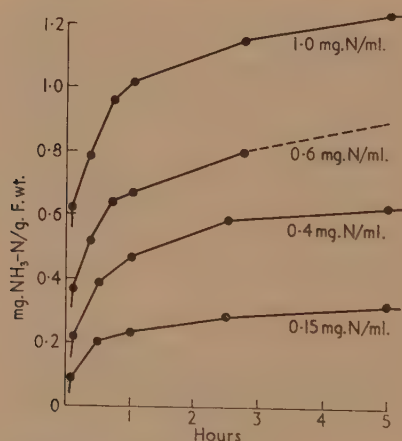


FIG. 4. Ammonia in mycelium of *S. brevicaulis* shaken in phosphate buffer (pH 7) containing ammonium sulphate at concentrations indicated

are greatest, but they are maintained for $\frac{1}{2}$ to 1 hour. Thus not only the equilibrium levels of ammonia in the cells, but also the rate at which ammonia enters is controlled by the ammonia concentration. These results all point to passive diffusion as the main process by which ammonia enters and is equilibrated in the cells.

The effect of pH. In the foregoing experiments the equilibrium was such that the internal concentration of ammonia (as $\text{NH}_4^+ + \text{NH}_3$) was close to the external concentration. These experiments were all done with an external pH 7, at which ammonia is almost completely (99.5 per cent.) ionized. Many plant cells are known to be readily permeable to the ammonia molecule, the

rate of diffusion into the cell depending on the concentration difference of the molecule in the two phases. At equilibrium the concentration of undissociated molecules inside the cell is thought to be the same as that outside (Davson and Danielli, 1952). The concentration of total ammonia (ions+molecules) in the cell, however, depends on the degree of dissociation, and therefore on the pH, in the cell. Thus when the internal pH is lower than the external the equilibrium concentration of total ammonia in the cells can be greater than the external. Such accumulation would not require expenditure of energy by the cell.

Present experiments with fungal cells are consistent with this view and an attempt to obtain quantitative experimental support has been made.

If the pH of the ammonia solution in which *S. brevicaulis* is shaken is raised to value greater than pH 7, thus increasing the proportion of ammonia in the undissociated form, the rate of uptake and assimilation rises with pH up to pH 8.5–9.0 (Morton and MacMillan, 1954). The higher rate of ammonia entry is not accounted for solely by more rapid assimilation, since during the first hour there is also an increase in the rate at which ammonia is accumulated (Fig. 5).

The equilibrium concentrations of ammonia in the mycelium at different external pH values are shown in Fig. 6A. The data are from a number of experiments in which the mycelium was extracted after shaking for 2–4 hours in buffers containing 0.4 mg. $\text{NH}_3\text{-N/ml.}$ (as $(\text{NH}_4)_2\text{SO}_4$). They show the marked increase in internal ammonia concentration as the external pH is raised. This effect of pH is also seen in the ammonia content of the cell sap (Table V). In this case the apparent ammonia concentration in the sap at high pH, where it is above the external concentration, is lower than the true value since the expressed liquid contains external solution for which no correction is made. That the accumulation of ammonia is a property of the living cells is seen from the results with cells killed by rapid heating at 100°C. prior to shaking with ammonia where no such accumulation was observed (Table V).

The expected internal equilibrium concentrations can be calculated for any given external pH and ammonia concentration but it should be stressed that quantitative considerations of this kind cannot be very precise. In the first place the internal concentration of ammonia cannot be accurately determined and is in any case probably not uniform throughout the cells. Secondly the 'internal pH' is a difficult measurement of uncertain significance, probably not uniform throughout the cell.

The proportion of undissociated molecules in the solution rises from about 0.5 to 35 per cent. between pH 7 and 9. If the pH inside the cells is unaffected

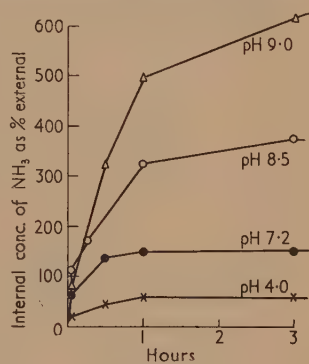


FIG. 5. Ammonia in mycelium of *S. brevicaulis* shaken in buffered solutions containing $(\text{NH}_4)_2\text{SO}_4$ (0.4 mg. N/ml.) at pH indicated

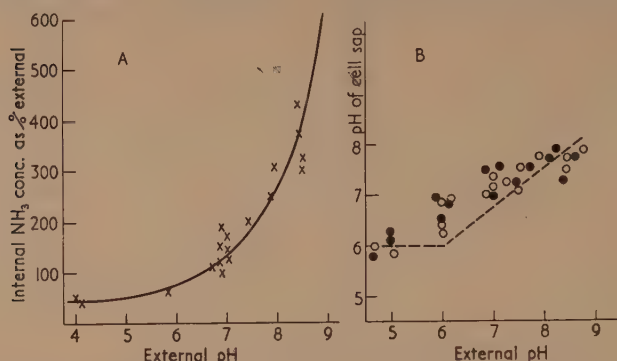


FIG. 6. Effect of shaking mycelium of *S. brevicaulis* in buffered solutions (0.4 mg. NH_3 -N/ml) on (A) ammonia content of the mycelium, and (B) pH of expressed cell sap; NH_3 supplied (●); no NH_3 (○). The dotted line shows calculated internal pH, see text.

TABLE V

Ammonia in expressed cell sap of mycelium shaken for 2 hours in solutions of $(\text{NH}_4)_2\text{SO}_4$ (0.4 mg. N/ml.) at different pH

	Living cells			Cells previously killed by rapid heating		
External pH . . .	3.7	6.9	8.3	3.1	5.1	8.2
NH_3 -N/ml. sap (mg.)	0.29	0.35	0.83	0.40	0.395	0.42
NH_3 -N/ml. in external solution (mg.) .	0.36			0.41		

by the changes in the external pH we would expect the total ammonia concentration in the cells to rise by a similar amount, but the 'internal concentration' of ammonia at pH 9 is found to be only six times as great as at pH 7 (Fig. 6A). If on the other hand the pH inside the cells rises with increasing external pH, so that the proportion of ammonia molecules in the cells is increased, then at high pH the expected concentrations of ammonia in the cell (as $\text{NH}_4^+ + \text{NH}_3$) would be lower and would agree more closely with those observed.

We have therefore measured the pH of the sap expressed from mycelium of *S. brevicaulis* after it had been shaken in buffered solutions for 2 hours. The mycelium was filtered off and freed from the buffer by washing in a large volume of distilled water before the sap was expressed. The results of several experiments (Fig. 6B) show that there is a definite rise in the pH of the sap with increasing external pH. It is interesting that the relatively large amounts of ammonia entering the cells, particularly at high pH, do not appear to alter the pH of the sap.

We have calculated internal pH values at which the observed internal ammonia concentrations would contain the same concentration of undissociated molecules as the external solution. These are shown in Fig. 6B. The

calculated values agree well with the observed pH values of the expressed sap but because of the complexity of the internal environment this agreement is perhaps of doubtful significance. But it does lend support to the view that the present findings agree in general with a passive equilibration of undissociated molecules.

Experiments with other fungi. The observations with *S. brevicaulis* have been compared with a few other fungi. The results with *Penicillium griseofulvum* (Fig. 7A) and with *Fusarium graminearum* (Fig. 7B) were very similar. Ammonia entry is rapid, and its rate depends on the pH of the external solution.

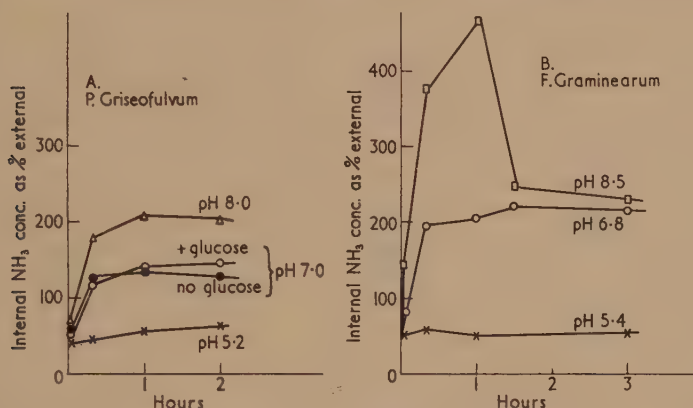


FIG. 7. Ammonia in mycelium of (A) *Penicillium griseofulvum* and (B) *Fusarium graminearum* shaken in buffers containing 0.4 mg. $\text{NH}_3\text{-N/ml}$.

Equilibrium levels, similar to those for *S. brevicaulis* were reached within 1 hour. With *F. graminearum* there is a sharp drop in the ammonia level at the highest pH after the first hour. This fact as well as the general appearance of the mycelium indicates that many of the cells are killed at this high pH. It is not known whether the high pH *per se* or the high internal ammonia concentration in combination with the high pH is the cause of death. The equilibrium levels of ammonia in this fungus are higher than in *Scopulariopsis* under the same conditions. This may mean that the internal pH of the cells is on the whole lower in *Fusarium*; certainly this fungus is less sensitive to low external pH (Morton and MacMillan, 1954).

Other experiments, with *Aspergillus niger*, have shown a similar rapid entry of ammonia, although a smaller effect of pH on the internal concentrations was observed.

DISCUSSION

It is widely held (Davson and Danielli, 1952) that ammonia, being a weak electrolyte, enters cells by the passive diffusion of its undissociated molecule. Osterhout (1935) and Jacques (1939) using cells of *Valonia*, found that the rate of entry of ammonia depends on the concentration difference of the

molecule between cell sap and external solution, and that the rate of entry was limited by the supply of certain cell constituents which combined with ammonia. More recently Äyräpää (1950) showed that yeast cells are very permeable to the ammonia molecule and there seems no doubt that in general penetration of undissociated molecules of weak electrolytes is largely determined by the concentration gradients (Simon and Beevers, 1952).

The present results clearly show that ammonia enters by passive diffusion, dependent on the concentration difference. The outward movement of ammonia can be as rapid as the inward, so that a dynamic equilibrium probably exists between the ammonia in the cell and the external solution, but this point requires confirmation by means of isotopic nitrogen. The inhibition of respiration does not affect the equilibration of ammonia in the cells at pH 7. The effect of respiratory inhibitors at higher external pH has not been tested but there would seem to be no grounds for supposing that a different mode of entry (i.e. an active transport) operates here. The accumulation of total ammonia in the cells against a concentration gradient of total ammonia at pH above 7 can be explained by the passive diffusion of the unionized ammonia molecule. The concentration of undissociated ammonia in the cell closely parallels that in the external solution over the wide range of pH used, if we assume that changes in external pH are accompanied by small changes in internal pH. This assumption is supported by the observation that the pH of the expressed cell sap does in fact change in this way. Similar flexibility of the internal pH of cells has been shown by other workers. Bünning (1936), using *Aspergillus niger*, found by means of indicator dyes an even greater response of the cell pH to changes in external pH. The entry and equilibration of ammonia in the form of the unionized molecule can thus account satisfactorily for the present results, including the accumulation against a concentration gradient at high external pH.

However, under metabolic conditions most of the ammonia is in the form of its ion and the possibility that the ions enter the fungal cells and at least in part determine equilibrium must be considered. The present experiments differ from studies such as those of Osterhout (1935) and Jacques (1939) which demonstrated the diffusion of the undissociated molecule of ammonia into the vacuolar sap of large-celled algae. In the present study the ammonia levels in the whole cell, i.e. cytoplasm as well as vacuoles have been measured and have been calculated as for a uniform internal environment. Evidence from the expressed sap indicates that the bulk of the ammonia is in solution in the cell, but we do not know how it is distributed between vacuoles and cytoplasmic fluid. However, the equilibration of ammonia appears to involve the greater part of the cell volume since the internal concentration is never much below the external.

It is known that in higher plants and algae metabolic energy is required to accumulate ions in the vacuole, but there is a growing body of evidence to show that they may enter the cytoplasm of higher plants and of bacteria passively by exchange reactions or by combination with a carrier (Broyer,

1951; Cowie and Roberts, 1954). Therefore it is possible that NH_4^+ ions enter the fungal cells, but we have no evidence to show whether their diffusion is restricted to any parts of the cell.

If diffusion of ions into the cells occurs, the equilibrium levels may be determined largely by a Donnan equilibrium between the external solution and the cell containing non-diffusible anions (Vervelde, 1953). This could account for a passive accumulation of ammonia against a concentration gradient. Ion exchange (Reichenberg and Sutcliffe, 1954) may similarly be concerned, since the necessary hydrogen ion gradient exists.

The present data could thus be consistent with entry of ammonia as ions or molecules; however, it can hardly be doubted that the ammonia molecule does penetrate the cells.

In some higher plant tissues the assimilation of ammonia can be as rapid as its entry into the cells, so that only traces of free ammonia are found in the cells (El-Shishiny, 1955). In the limited range of fungi studied here the entry of ammonia is more rapid than assimilation. The ammonia content of the cells is therefore directly dependent on the ammonia in the external solution even during active growth and the process of assimilation is not limited by the entry of ammonia into the cell.

ACKNOWLEDGEMENT

I wish to thank Mr. D. H. W. Scott for skilled technical assistance.

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Distribution of Growth in the Apical Region of the Shoot of *Lupinus albus*

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Received 15 August 1955

SUMMARY

The purpose of the investigation is the determination of the volumes and numbers of cells of the meristematic dome and of each of the first 7 primordia and internodes at the apex of the shoot of *Lupinus albus*. This system occupies a zone which is about 0.4 mm. in length. Techniques are described for dissecting the region in which the observations are made, for determining the numbers of cells and the volumes of the several fragments. From the number of cells and the volume of each fragment an average cell volume is calculated.

It is shown that in the midphase of the plastochron the dome contains 3,500 cells and has a volume of 1.6×10^{-3} mm.³, the first primordium contains 1,630 cells and has a volume of 0.38×10^{-3} mm.³, and the first internode contains about 700 cells and has a volume of about 1.4×10^{-3} mm.³ The number of cells and the volume of the primordium increase exponentially with increasing plastochron age, and the seventh primordium contains 26,000 cells and has a volume of 20.9×10^{-3} mm.³ The seventh internode contains about 5,000 cells and has a volume of 8.6×10^{-3} mm.³

The average cell volume in the dome is 4.7×10^{-7} mm.³, in the first primordium it is 2.3×10^{-7} mm.³ and in the first internode it is 20.9×10^{-7} mm.³ In the seventh primordium the average cell volume increases to 7.9×10^{-7} mm.³ In the internodes there is little, if any, change in cell volume from the first to the seventh of the series.

The significance of these changes is discussed.

INTRODUCTION

THE purpose of the investigation, the results of which are reported here, is the interpretation of growth in the embryonic region of the shoot in terms of the formation and subsequent expansion of constituent cells. The observations have been restricted to a region which is 0.4 mm. in length and which includes the meristematic dome and seven primordia each attached to an internode of the central axis. Within this region tissue differentiation is limited and the whole system is in that sense embryonic. The investigation has been designed to provide data on the volumes of the different parts of the system and on the numbers and volumes of cells in each part.

The morphological and histological aspects of the events that occur in the apex of the shoot have frequently been described. The quantitative aspects of the changes involved have not, however, been extensively examined. These are likely to be of some significance in two connexions. The quantitative

changes that accompany the induction and subsequent growth of primordia are likely to be important limiting conditions in the development of the morphological pattern. Secondly, the growth that proceeds in the apex establishes the basis from which growth in the non-embryonic phase must continue and it is therefore an important factor in the determination of the growth of the shoot as a whole. It is clear, however, that whatever the context in which the growth determination is made, simple volume or weight measurements are of restricted significance. Growth in this, as in all other parts of the plant, is an expression either of an increase in the number of cells or of an expansion of constituent cells. The mechanisms involved in the two processes are different and the significance that is attached to changes in either weight or volume must vary according to the nature of the changes in the primary cellular conditions.

The general design of the investigation is based on the fact that leaves and internodes are generated serially from the meristematic dome. The basipetal succession represents therefore a succession of increasing age, and measurements made on the successive members may be taken as showing the changes promoted by growth with time. As indicated above, all the observations of this series have been made on a single fragment. This has been excised from seedlings all at the same developmental stage. The fragment has been dissected in such a way that values are obtained for the dome, each primordium and each internode. These successive values have been taken here as representing the changes in a single entity with time. It is recognized that this approach carries one serious limitation. The interpretation of the successive primordium values for instance in terms of the development of a single primordium requires the assumption that the initial primordium mass is always the same. For this there is no evidence and indeed there is some indication to the contrary. On the other hand variation in the initiating mass is likely to be small relative to the overall changes that occur with development, and it is probable that the general procedure adopted here does provide an indication of at least the general features of embryonic growth.

This analysis of growth in the shoot apex is similar to that which has already been applied in the root (Brown and Broadbent, 1949; Erickson and Goddard, 1951). It differs from it, however, in one important respect. The determination of volumes and numbers of cells is not made at determinate and arbitrary distances from the apex, but at positions that are determined by the morphology of the system. In certain respects this restricts the value of the data. On the other hand, it carries the valuable advantage that through it the time scale involved in the changes measured may be estimated at least approximately. The interval between the production of successive primordia, while it undoubtedly varies, tends to fluctuate about a mean value, and when this has been determined the rates of the several changes may be calculated.

The techniques that have been used are described below. Two general features of the situation to which the techniques must conform may be noted here. The fragments are small (the smallest has a volume of about

0.8×10^{-3} mm.³) and since the excision of a single apex necessarily involves the use of a seedling about 3 weeks old, the number of fragments available for any single determination must be limited. In this situation, conventional techniques which depend on milligram quantities are inapplicable. For routine work they must be such that they can be used with microgram quantities which involve either a single or a limited number of apices. Secondly, the tissues are extremely delicate. They are very susceptible to injury when normal manipulative techniques are used. It is important, particularly when the preparations are required for metabolic studies, that frequent contact with dissecting instruments be avoided.

MATERIALS AND METHODS

The investigation is based on apices taken from seedlings of *Lupinus albus*. This species has been chosen since it provides a comparatively large meristematic system, which is readily exposed, and it has already been used in a number of other investigations.

The apices are taken from seedlings that have been grown in boxes in a frame. During the winter months the frame is heated, although the temperature is not rigidly controlled and the seedlings are provided with supplementary illumination after sunset. The apices are excised from plants which are between 3 and 4 weeks old and which carry a total of 17 to 19 primordia and fully expanded leaves. At this developmental stage the plant is fully vegetative and is still producing only leaf primordia and stem internodes from the meristematic dome.

Design of the investigation. As indicated above the investigation requires the determination of the total volume, the number of cells, and the average cell volume of the dome, and of each of the first 7 primordia and 7 internodes.

The general design is based on determinations of numbers of cells and volumes on three types of fragments. Average cell volumes are calculated from the two primary determinations. The measurements are made after dissecting an intact fragment into smaller units. The intact system, however, is not dissected into its several constituent morphological components, and the values for certain parts of the system are not obtained by direct determination.

In principle from embryonic systems excised from the apices of seedlings three types of fragment are derived (1) apical domes, (2) primordia, and (3) apical units.

The characteristics of each type of fragment are shown in the diagram of Fig. 1. The apical dome *D* is the region above the level of the first primordium. The primordia are, of course, the structures which subsequently develop into leaves, and in the diagram they are marked P_1 to P_7 . P_1 is the first primordium in the series and is that immediately below the dome. P_7 is the seventh primordium and is that most remote from the apex. Apical units are systems which consist of a variable number of primordia attached to a central axis which is terminated by the meristematic dome. Apical units are

enumerated as units 1, 2, and 3 up to 7 according to the number of primordia that they carry. In the diagram the characteristics of units 2, 5, and 7 are shown. Each carries a dome. Unit 2 in addition to this involves the first two primordia, P_1 and P_2 , and the first two internodes I_1 and I_2 . Unit 5 includes in addition to the structures of unit 2, the third, fourth, and fifth primordia, P_3 , P_4 , and P_5 and the corresponding internodes I_3 , I_4 , and I_5 .

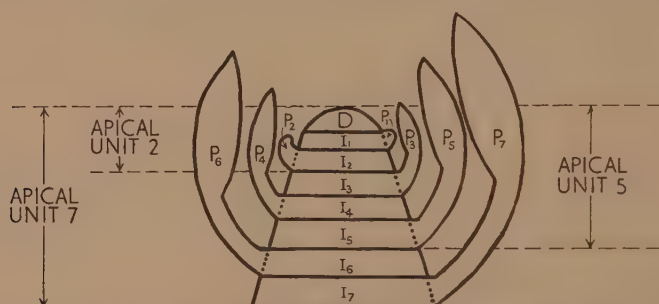


FIG. 1. Diagram of embryonic region analysed, showing dome D , internodes I_1 to I_7 , primordia P_1 to P_7 , and apical units 2, 5, and 7.

The dome is readily excised and direct observations are made on it. All the primordia, except the first, are also readily excised and again observations are made directly on them. The first primordium cannot be removed since the limits between itself and the axis to which it is attached are not well defined. Values for the first primordium are obtained indirectly by a procedure that is given below. The values for the separate internodes are obtained from apical units. The difference between the values given by two successive units in the series represents the contribution of one primordium and its associated internode. The value due to the primordium has been separately determined, and when this is deducted from the total difference a value for the internode is obtained. This general technique for the determination of internode values is open to the objection that they are derived from three sets of measurements and therefore carry the corresponding number of errors. It is, however, probably preferable to the alternative of excising each internode separately. The internode is seldom more than 30μ thick and excision at these limits would necessarily involve destroying a large proportion of the cells in the structure. Internodes are enumerated in the same order as the corresponding primordia. In the diagram of Fig. 1, the first which is that immediately below the dome is marked I_1 , and the seventh which is that most remote from the dome is marked I_7 .

The difference between the values for two successive apical units is the quantity from which the internode value is obtained. This quantity, however, is also of some significance in other connexions, and it is distinguished here as a *growth unit*. It represents a complex of a primordium and the corresponding internode, and the different growth units are enumerated in the same order

as the primordia and internodes of which they are composed. The first growth unit is that which is immediately below the dome and is constituted from P_1 and I_1 . The seventh unit is that most remote from the dome and is constituted from P_7 and I_7 . It may be emphasized that the term growth unit carries no morphological implications, and it is used here only with respect to the requirements of a particular analytical situation.

One further term that is used below requires to be defined. The term plastochron is used in the conventional sense. Plastochron age, however, refers to relative age corresponding to position in the serial order. Thus the primordium, internode or growth unit, which is third in the series, has a greater plastochron age than that which is second and a smaller one than that which is fourth.

In certain instances the variability with duplicate determinations is high. Determinations of volume with corresponding primordia taken from different plants may for instance vary by 200 per cent. Accordingly corresponding observations have been made on groups of fragment. In most cases within the group each fragment has been measured separately. In certain cases, however, observations have been made on 2 or 3 fragments simultaneously. The values given below are the means from a set of measurements on 8 to 10 fragments.

The large differences between duplicate determinations are due primarily to variability in the material itself. They are, however, also partly due to two errors involved in the method of selection of the parent material. There may be some difficulty in identifying a particular primordium. The enumeration begins from the first and when this is small it may be overlooked and a primordium may then be assigned to a position higher than that which in fact it occupies. Also although a primordium occupies the same position throughout a plastochron its size is likely to increase during the course of it, and a plant taken at the beginning of a plastochron is likely to give lower values than one taken at the end.

The techniques which are described below are required in each of the following phases of the investigation: (a) excising the embryonic region from the rest of the seedling, (b) dissecting the embryonic region into apical units, dome, and primordia, (c) determining the number of cells in each of the fragments, and (d) determining the volume of each excised fragment.

(a) *Excising the embryonic region.* As indicated above apices are excised only from seedlings having a total of about eighteen leaves and primordia. From the selected plants the leaves are stripped from the stem beginning with the lowest and proceeding upwards until the embryonic region has been exposed. At this stage the stem at the apex has the appearance of a flat cone to the centre of which a short column is attached. This column carries the embryonic region at its apex and it is excised by four cuts made longitudinally into the stem. The cuts outline a square with the embryonic region in the centre and they isolate a strip of mature tissue which carries this region apically. The cuts are dispersed so as to make the strip wider than the terminal column.

The strip is detached from the stem by pinching it off with forceps at about the limit of the longitudinal cuts. The excision of the embryonic tissue in this way is important since it provides a means of manipulating the material without touching it.

(b) *Dissecting the excised fragment.* From the material removed from the stem an apical unit, a dome or a primordium may be required. These are obtained by cuts or dissections at appropriate levels made after the tissue on the excised column has been partially embedded in a suitable medium. Embryonic tissues are extremely delicate and are extensively deformed when they are cut without the support of an embedding medium. The normal biological embedding techniques cannot be used since with these the tissue must be exposed to a comparatively high temperature. The medium must be such that it has no toxic or dehydrating effects and it must also have little or no elasticity. A large variety of materials has been tried, but only one, clay, has given satisfactory results. From a clay preparation tissue can be excised relatively accurately in an undeformed state. The tissue so obtained can be used for respiratory studies, for chemical analysis, and for volume and cell number determinations. Clay, however, has one disadvantage. If the water content is high, particles of clay may cling to the tissue, and these cannot be removed by washing. This is avoided by using clay which has approximately the consistency of firm plasticine and by regulating the depth to which the fragment is embedded. If the tissue is not required for metabolic studies the difficulty may also be avoided by adding a drop of hydrocarbon oil to the clay. Either potter's or modelling clay may be used. All the work of this investigation has been done with a single sample of potter's clay.

The embryonic material on the strip of stem tissue is transferred to a clay pad about 1.0 cm. in diameter and 2.0 mm. deep on a microscope slide. Pressure is applied to the strip of mature tissue and this sinks into the clay. The strip, which is considerably thicker than the column, is firmly embedded before the embryonic region comes into contact with the clay. The pressure on the strip is maintained until the lower half of the embryonic mass has also started to sink into the embedding medium.

At this stage a dome or an apical unit may be excised from the preparation. If the dome is required, before embedding, the embryonic mass is arranged with the first primordium to one side of the preparation. In the case of the apical unit the mass is aligned on the clay surface with the leaf base that defines the limit of the unit again at one side.

Cutting is done with a small strip broken from a safety-razor blade and gripped in a suitable holder. The point of the strip is thrust into the clay to one side and the cutting edge is drawn across the preparation at the appropriate level. If a dome is being excised the cut is made immediately above the first primordium; if an apical unit, the cut is made through the appropriate leaf base.

The procedure used in the excision of the primordium varies with position. The second and third are excised from apical units 2 and 3. Each of these is

transferred from a horizontal to a vertical position with the cut surface resting on the clay. The primordium is then removed with a vertical cut in the angle between the primordium and the axis. The fourth and lower primordia cannot be excised by a single cut since with these the stipules have been extensively developed and the base occupies a considerable proportion of the circumference of the axis. It has been found, however, that the whole structure may be removed by thrusting a sharp point between one stipule and the axis and then more or less peeling the primordium away.

All the operations in the sequence after the removal of the fully expanded leaves from the selected seedlings are done with a dissecting microscope. All the work has been done using a binocular microscope equipped with stage, $\times 14$ eyepieces, and $\times 5$ paired objectives.

It is recognized that the general technique of dissection does not yield fragments that are separated from the rest of the system at well-defined morphological limits. The dome is excised by a cut at right angles to the long axis. It may, however, be that the first growth unit is delimited from the dome in an oblique plane, and if this is the case then the technique used may yield exaggerated values for the first growth unit. Secondly, when the second and third primordia are excised it is possible that portions of the stipules are left on the central axis, and when the fourth and subsequent primordia are removed it is possible that portions of internode tissue are torn away along with the foliar structure. Finally, the technique of dissection gives an internode which stretches from one leaf base to the next. There is, however, no evidence which indicates that the mature structure is developed from an embryonic zone with these limits. At the same time although the technique may not yield data which refer to precise morphological regions, it undoubtedly provides data which show the general quantitative characteristics of the system.

(c) *Determining the number of cells.* In an early phase of the investigation attempts were made to apply the same general technique as that used by Brown and Rickless (1949) with root sections. It was found, however, that macerating fluids (and several were tried) tended to disintegrate the cells. The maceration technique depends in part on the presence of a relatively firm cell-wall system, and this condition is apparently not available with shoot apices. Also with fragments that are only just visible to the naked eye, losses occur fairly frequently, and when the numbers are limited this introduces a serious error.

Another technique has therefore been developed which depends on a different principle. Single fragments, after being treated in such a way that the attachment between the cells is broken without disorganizing the cells themselves, are spread on a microscope slide into a plate, one cell thick with the cells more or less separated from each other. The cells are counted in transects of known width taken at intervals across the plate, the intervals being constant multiples of the transect width. The number of cells counted is multiplied by the multiple of the transect in the interval and this gives the total number of cells in the fragment.

Immediately after excision the tissue is treated first with acetic alcohol for 10 minutes and then with normal hydrochloric acid for 8 minutes at 60° C. The acid is removed and replaced by a 1 per cent. methyl-green solution to which sufficient sodium bicarbonate has been added to make it alkaline. The tissue is left in the stain overnight. The length of the treatment in the acid is critical and the alkaline condition in the stain is important. With prolonged exposure to even weak acids the cell contents tend to disperse and with a tissue impregnated with acid fluids the cells do not separate without folding.

All the above treatments are applied on a single cavity slide, with the tissue in the cavity. Fluids are removed with strips of filter paper and applied with a fine pipette. During treatments the slide rests on the rim of a watch glass which stands in a petri-dish containing the same fluid as that on the slide. When the dish is closed this arrangement prevents evaporation from the small volume on the slide.

When staining is complete the fragment is transferred to a drop of 50 per cent. glycerol on one of the flat surfaces of the cavity slide. If the preparation is now covered with a glass cover slip and pressure applied to it through the glass the tissue disintegrates into an irregular plate which may be several cells in depth. With this condition counting is impossible. The difficulty is avoided, however, by using a plastic instead of a glass cover slip. A strip about the size of a standard cover slip is cut from a thin pliable and transparent plastic sheet. Pressure is applied to the tissue in the glycerin through this strip with a fine glass rod which has been rounded at one end. The surface of the plastic is rubbed until cell clumps have been dispersed and the cells have been separated from each other. The glycerol solution prevents erratic dispersal as a result of the rubbing. If a less viscous fluid is used the cells may be spread over the whole area under the coverslip. With glycerol on the other hand they tend to remain in a fairly restricted patch.

All the operations to this stage are performed on the stage of a dissecting microscope. It is particularly important that the preparation be observed microscopically during the preparation of the cell plate. With this provision, the general effects of rubbing on the surface of the plastic can be controlled.

When the dispersal of the cells is complete the preparation is transferred to the mechanical stage of a monocular microscope equipped with a $\times 10$ eyepiece and $\times 40$ objective. The eyepiece carries a disk marked in uniform squares, and it is rotated until the squares are aligned in north-south and east-west directions. Two parallel lines running north-south are chosen to define a transect between them. The microscope stage is now moved in an east-west direction until an extreme lateral edge of the cell plate is visible between the transect lines. In this position, counting begins. The stage is moved in a north-south line, and when the plate is no longer visible the direction of movement is reversed and all the cells that fall between the transect lines are counted. When the whole plate has been traversed, still in the same line, the plate is brought back to the centre of the field, and it is now moved

laterally through a certain number of transect widths. A cell which is lying against one of the transect lines is chosen as a marker and it is moved to the same relative position against the second line. A second marker cell is chosen and the process repeated as many times as the situation requires. The plate is now in the second counting position and again it is moved in a north-south direction and again the direction of movement is reversed when no cells are visible. After reversal the cells are counted as before. The whole procedure is repeated until the entire cell plate has been covered. The number of cells counted in the several transects is multiplied by the number of transect widths through which after each counting the preparation is moved laterally. This gives the total number of cells in the preparation. Counting is facilitated by the staining which imparts a deep blue colour to the cells.

When the cells have been satisfactorily dispersed the irregularities in the plate are not large, and the proportion of the number of cells counted can be comparatively low. Table I shows the results obtained when a number of determinations are made on a single preparation with different fractions of the whole cell plate covered. It is evident that the result is approximately the same when half and when only one-sixth of the field is covered. These results also show that the errors of the method are of the order of 15 per cent.

TABLE I

Determinations of numbers of cells in two preparations with different fractions of whole preparation covered

$\frac{1}{2}$.	.	.	7278	6914
$\frac{1}{3}$.	.	.	7569	7302
$\frac{1}{4}$.	.	.	8100	7432
$\frac{1}{5}$.	.	.	7700	6485
$\frac{1}{6}$.	.	.	7482	

In this work the proportion of the total covered has varied between $\frac{1}{4}$ and $\frac{1}{7}$, the higher proportion being used with the smaller preparations since the errors due to irregular dispersion are likely to be greater with these.

(d) *Determination of Volume.* The principle of the method is simple. The tissue is compressed between glass plates that are screwed together but separated at their edges with thin spacing pieces. The area into which the tissue is flattened is measured and from this and from the known thickness of the spacing pieces the volume is calculated.

The two glass plates are of the same size as standard microscope slides, although considerably thicker. It was found that thick glass is essential since even with small tissue fragments microscope slides tend to buckle. The spacing pieces are placed between the ends of the two plates and they are each about 2 cm. square. Originally metal sheets were used. Later it was found that strips of cellophane were more convenient and in certain respects more satisfactory. When these are used they give a free space midway between the ends of the two plates of about 24.5μ . The glass plates are clamped together at their ends by two pairs of metal disks which are wider than the

plates and which are drawn together by screws through their overlapping edges.

An important feature of the technique is that the fragment is compressed in a drop of paraffin oil. During compression water tends to be expelled from the cells, and when oil is present it does not escape but remains closely associated with the flattened tissue. The limits between the oil and the water and tissue system are, of course, sharply defined by the difference between the refractive indices of the two fluids.

The area of water and tissue is determined from a camera lucida drawing of the outline of the flattened mass. The magnification at which this has been drawn is readily determined, and when the area within the outline has been measured, the area of the original mass may be calculated.

The errors of the method are given by the technique of calibration. A small drop of mercury of known weight is compressed between the two glass plates in the normal manner. The area that this forms is also determined by the technique outlined above. From the weight and from the known density of mercury the volume of the drop is calculated and from this and the area of the compressed drop the width of the gap between the two glass plates is estimated. Six determinations of the gap width with the same cellophane spacing pieces gave 24, 24, 24, 25, 25, and 25 μ . Thus the error with any single determination is less than 5 per cent.

RESULTS

The results are presented under the following headings: (a) Apical Units; (b) Growth Units; (c) Primordia; and (d) Internodes. The data for the dome are included with those for apical units. In each connexion values are given for total volumes, total numbers of cells, and average volumes of cells. It may be emphasized that the primary data are those for volumes and cell numbers determined with the dome, apical units and primordia. The values for growth units and internodes are derivative data and in all cases the figures given for average cell volumes are calculated values.

Apical units. The volumes, numbers of cells and the average cell volumes of the dome and of the successive apical units are shown in Table II.

TABLE II

Values for dome, D, and for successive apical units. Total volume V ($\times 10^{-3}$ mm.³); number of cells N ; and average cell volume CV ($\times 10^{-7}$ mm.³)

Fragment	V	N	CV
D	1.6	3,500	4.7
1	3.4	5,800	5.9
2	5.6	10,100	5.6
3	8.9	15,400	5.8
4	15.7	25,300	6.2
5	28.9	39,600	7.3
6	46.8	61,400	7.6
7	76.3	92,900	8.2

The successive apical units carry an increasing number of primordia and internodes, and the increases in number of cells and volume as the order is traversed corresponds to this condition. The seventh unit which has a volume of $76.3 \times 10^{-3} \text{ mm.}^3$ and involves about 93,000 cells may be taken to represent the growth that is made during seven plastochrons by a system that starts from an apical dome with a volume of about $1.6 \times 10^{-3} \text{ mm.}^3$ and carrying about 3,500 cells. The increments in volume and numbers of cells between the initiating dome and the seventh unit is, of course, not due only to the addition to the system of another growth unit during each plastochron. The data of Tables III, IV and V show that the development of an additional growth unit is accompanied by increases in volume and numbers of cells in the older components of the system. Thus, the values for the seventh unit represent the resultants of successive growth units contributed to the system and of progressive volume and cell number increases in all parts of the system during the successive plastochrons.

The data given in the last column of Table II show that as the serial order is traversed the average cell volume increases. It increases from about $4.7 \times 10^{-7} \text{ mm.}^3$ in the dome to about $8.2 \times 10^{-7} \text{ mm.}^3$ in the seventh apical unit. This change indicates that as the tissue matures the component cells enlarge. This is also shown by the data of Table III. The average volume increase with increasing serial order is, however, smaller than that found in the growth units and primordia with increasing plastochron age. This difference is due to the condition that the apical unit involves tissues of different plastochron ages, and the average value for each unit is, therefore, determined by groups of cells in different stages of development and consequently of enlargement.

Growth units. The volumes, numbers of cell and the average cell volumes of the successive growth units are shown in Table III.

TABLE III

Values for successive growth units. Symbols and quantities as in Table II.

Fragments	V	N	CV
1	1.8	2,300	7.6
2	2.2	4,300	5.1
3	3.3	5,300	6.2
4	6.8	9,900	6.9
5	13.2	14,300	9.2
6	17.9	21,800	8.3
7	29.5	31,500	9.4

A comparison of the data for the first growth unit given in Table III with those for the dome given in Table II shows that the unit has approximately the same volume although it contains a smaller number of cells than the dome from which it is formed. The average cell volume in the first unit is larger than it is in the dome.

It is evident that volume increases with increasing plastochron age. The volume increase from the first to the seventh unit being about 15-fold. The

data indicate that the increase is due primarily to cell division. Whereas, from the first to the seventh unit the increase in average cell volume is less than double, the increase in cell numbers is about 15-fold. It may be emphasized that each growth unit represents a primordium and the corresponding internode, and each overall value is therefore a mean from the two components. It is to this that the considerable differences between the average cell volumes of the primordia and internodes on the one hand and of the growth units on the other hand are due.

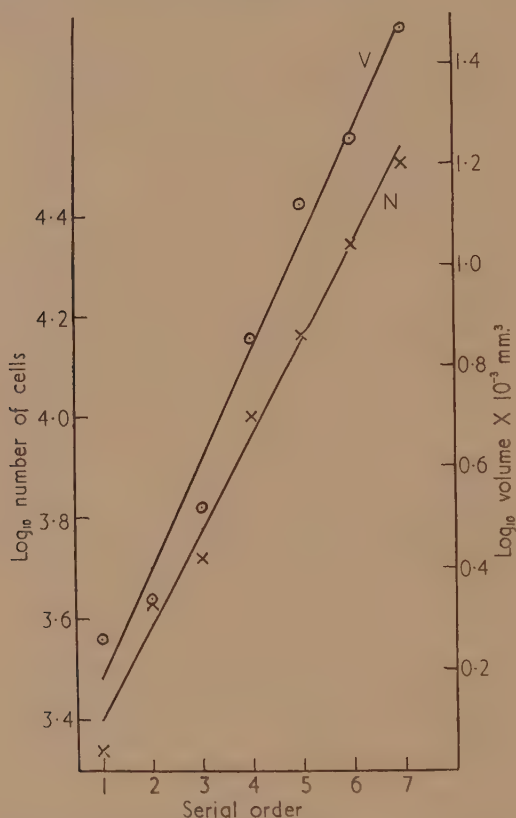


FIG. 2. Log₁₀ values of volumes *V* and numbers of cells *N* of successive growth units.

An important feature of the data is shown by Fig. 2 in which the logarithmic values for volumes and numbers of cells are plotted against the serial order. It is evident that at least the numbers of cells increase exponentially with increasing plastochron age.

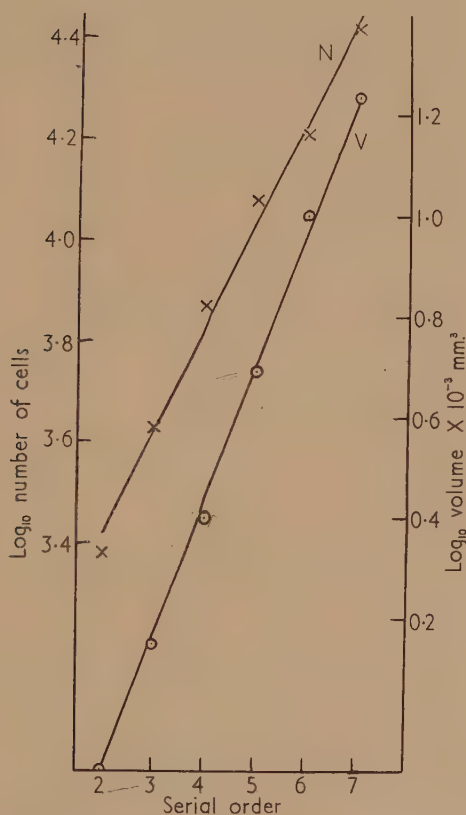
(c) *Primordia*. The volumes, numbers of cells and the average cell volumes of the second and subsequent primordia of the series are shown in Table IV.

The logarithmic values for cell numbers and volumes have been plotted against the serial order in Fig. 3. It is evident that in each case the increase

TABLE IV

Values for successive primordia. Symbols and quantities as in Table II

Fragment	V	N	CV
1	(0.38)	(1,630)	(2.3)
2	0.8	2,400	3.2
3	1.4	4,300	3.4
4	2.5	7,400	3.4
5	4.9	12,000	4.1
6	10.0	16,300	6.1
7	20.9	26,400	7.9

FIG. 3. Log₁₀ values of volumes *V* and numbers of cells *N* of successive primordia.

is exponential with increasing plastochron age. The regression equation to the line of closest fit for the cell number data is: $Y = 3.01 + 0.204 X$ and for the volume data it is $Y = 1.30 + 0.284 X$. As indicated above direct determinations on the first primordium cannot be made. However, it is significant that the cell-number data for growth units increase exponentially from the first to the seventh. The determinations for growth units and

primordia are made with different fragments and are therefore independent. At the same time the data indicate that the number of cells from the primordium in the growth unit is always greater than that from the internode. Thus the exponential increase in the growth unit could only occur if the primordium values increase in the same way, and the results obtained with the units may thus be taken to indicate that cell numbers in primordia increase exponentially from the first. If this is the situation with cell numbers then it is certainly also the case with volumes. Accordingly the volume and cell-number values for the first primordium have been calculated from the regression equations and are entered in Table IV in brackets.

The values for the first primordium may be compared with those for the first growth unit given in Table III. It is evident that whereas the volume of the primordium only represents about 20 per cent. the number of cells in it represent about 70 per cent. of the first unit. These differences correspond to an average cell volume which is smaller than that for the unit as a whole. At the same time it is significant that the average cell volume is smaller than it is for the dome as a whole.

The volume of the primordium increases considerably with increasing plastochron age. From the first to the seventh the enlargement is about 50-fold. In this case this is due partly to a threefold increase in the average cell volume. On the other hand it is clear that the growth of the primordium is determined primarily by cell division, since from the first to the seventh the number of cells increases about 20-fold.

Internodes. The volumes, numbers of cells and the average cell volumes of the successive internodes are shown in Table V.

TABLE V

Values for successive internodes. Symbols and quantities as in Table II

Fragment	V	N	CV
1	1.4	670	20.9
2	1.4	1,900	7.6
3	1.9	1,000	19.0
4	4.3	2,500	17.2
5	7.8	2,300	33.6
6	8.5	5,500	15.5
7	8.6	5,100	16.8

It has been shown above that the error involved in the estimation of these data is likely to be large. Nevertheless, the groups involve large differences which indicate important characteristics of the growth of the whole system.

A comparison of the values for the first internode with those for the first unit given in Table III shows that whereas the internode contributes about 80 per cent. of the volume, it only contributes about 20 per cent. of the cells of the internode. This corresponds to the converse situation with the primordium. The average cell volume in the first internode, however, is not only larger than it is in the first primordium but also larger than it is in the dome.

It is evident that volume increases with increasing plastochron age. From the first to the seventh internode the enlargement is about 6-fold. The increase in the number of cells is of the same order, and it is therefore probable that the increase in volume of the internode is due to division, with the maintenance of an approximately constant cell volume. This interpretation is consistent with the cell-volume data, which although variable do not indicate any consistent increase.

DISCUSSION

For the interpretation of the significance of certain of the results presented above, an estimate of the average duration of the plastochron is required. This is obtained by counting the number of foliar units in seedlings of different ages; and the results of one set of determinations are given in Fig. 4.

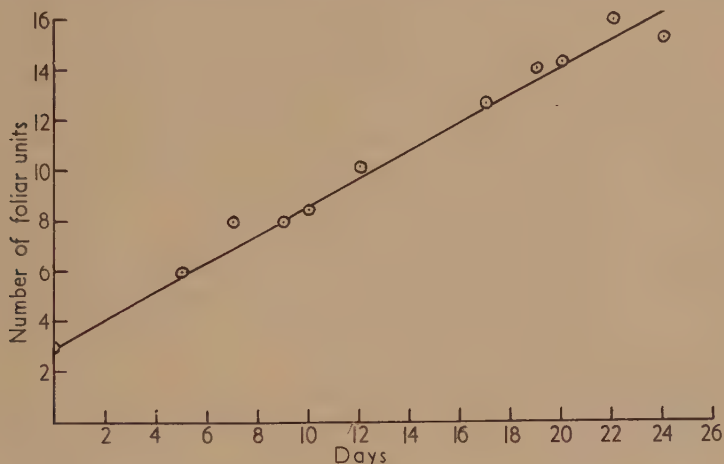


FIG. 4. Numbers of foliar units plotted against seedling age.

The data suggest that while the rate at which primordia are produced may fluctuate considerably, nevertheless, they tend to be produced with an average frequency of about one every 2 days. In this connexion it is important to notice that in this investigation the seedlings were not grown in constant conditions and it is probable that the duration of the plastochron along with the rates of other phases of growth varied considerably.

The largest apical unit included in this series of observations carried 7 growth units. This system may be taken to represent the growth made during the course of 7 plastochron intervals from an initiating dome. This apical unit has a volume of about $76.0 \times 10^{-3} \text{ mm}^3$ and this corresponds to a fresh weight of about 0.076 mg. Since the average duration of the plastochron is 2 days this mass is the product of growth during about 14 days.

The absolute rate of growth in the embryonic region of the shoot is clearly very low. It is so, of course, partly as a result of the small mass of tissue from which the growth begins. The volume of the dome is only $1.6 \times 10^{-3} \text{ mm}^3$.

which corresponds to a fresh weight of about 0.0016 mg. However, the low absolute rate is also undoubtedly a consequence of slow rates of division and cell expansion. The rate of division may be calculated from two sets of data. It is shown below that at the beginning of a plastochron the numbers of cells in the dome and in the first growth unit are probably smaller than those indicated by the data given in the last section. The ratio between the two will, however, be the same, and it may be taken that a dome with 3,500 cells during a plastochron of 2 days produces a growth unit carrying about 2,300 cells. This suggests for the dome an average rate of about one division every third day. Secondly, after 7 plastochrons and therefore after about 14 days the growth unit contains about 32,000 cells. These start from a unit which involves 2,300 cells, and this indicates that the average rate of division in the unit is of the same order as it is in the dome. Richards (1951) estimated the average rate in the meristem of *Lupinus albus* to be one division every 5 days. The value given here is slightly greater than this but of the same order, and supports the conclusion that the average rate of division is low.

It is probable that the average cell volume in the growth unit approximately doubles between the first and the seventh. If the average cell volume remains constant then in a system in which division is occurring this involves a doubling in volume during each cell generation. If the average increases this implies something greater than a twofold increase, the excess depending on the number of cell generations involved in the period during which the average increases. Each generation occupies about 4 days and in the 14-day period there are about 4 generations. This suggests that during each period of 4 days the volume increase is about 2.4-fold.

The position in the apex of the shoot with respect to division and expansion may be compared to that in the root. Brown (1951) has shown that at about 20° C. in the pea root the overall cycle in division occupies about 18 hours, and the data of Erickson and Goddard (1951) indicate that in the maize root a ten-fold increase in cell volume is completed in about 12 hours. Clearly the division and expansion probably proceed considerably more slowly in the embryonic region of the shoot than they do in the corresponding regions in the root.

The rate of about one division in 3 days is, of course, an average value. It does not imply that all the cells in the dome divide at the same rate or that all the cells in each growth unit divide at the same rate. On the contrary some of the data considered below indicate that the average rates of division in the internodes and in the primordia differ considerably. At the same time it is probably a feature of some significance that the average rates in the dome and in the rest of the system are approximately similar. Evidence that is presented elsewhere indicates that the cell expansion that occurs during development is not an expression of increasing protein but is due to an increasing water content with consequent vacuolation. This is evidently extensive and the data suggest that at least in the embryonic region of the shoot vacuolation as such has little effect on division.

The values given above for the numbers of cells and volumes of different parts of the embryonic system represent the position in the middle of a plastochron. The values are based on random samples which contain seedlings in all stages of development with respect to a particular plastochron. The sample is therefore likely to contain some seedlings giving low values for the early stages of development and others giving high values for the later, and the mean therefore represents the intermediate state when the seedling is in the middle phase of development. At the beginning of the plastochron the values are likely to be smaller and at the end they are likely to be larger than those recorded, although relative differences between different parts of the system will be the same whatever the stage of development.

The changes in the dome may be considered in terms of an expansion which starts at the beginning of a plastochron and which continues for about 2 days. At the end of this period from the expanded mass a new growth unit is delimited. For the reasons given above the absolute number of cells in the dome and in the unit immediately after differentiation cannot be determined from the data available. In the following plastochron, however, it is probable that the rate of cell division in the two is the same, and it is therefore probable that the proportion between the cells of each in the middle of this plastochron is the same as it was at the beginning. The present series of data indicates that in the midphase the number of cells in the dome is 3,500 and in its product it is 2,300. This suggests that when differentiation occurs between 35 and 40 per cent. of the cells in the dome are contributed to the new growth unit.

The cells in the young growth unit immediately after differentiation are distributed between the primordium and the internode in the ratio of 16:7. As the unit ages the ratio between the two increases. In the seventh plastochron the ratio is about 31:5. The change in the proportion of cells in the two components of the unit is clearly due to different average rates of division in the two. In the internode the number of cells changes in the course of 7 plastochrons from about 700 to about 5,000, and in the primordium from about 1,600 to about 26,000. These changes correspond to average rates of about one division every 5 days in the internode, and about one division every 3 days in the primordium.

At the time of initiation not only the numbers but also the average volumes of the cells in the two components of the first growth unit are different. The average volume in the primordium is about 2.3×10^{-7} mm.³ and in the internode it is about 17×10^{-7} mm.³ The average in the internode is clearly about 8 times greater than it is in the primordium. This difference is of some significance in relation to the tissues in the dome from which the two structures are differentiated. The average volume in the dome is 4.7×10^{-7} mm.³ It is clearly improbable that during half a plastochron the rate of division in the primordium is sufficiently rapid to reduce the average volume to half that in the dome or that the rate of expansion in the internode is sufficiently rapid to increase the average volume to 4 times that of the dome. The average

for the dome is, of course, a mean of the volumes of different types of cells, and it is therefore probable that the relatively small average volume in the primordium is due to formation from a tissue in the dome composed of small cells. Similarly the large average value for the internode suggests that it is formed from a tissue in the dome composed of relatively large cells. It is of some significance in this connexion that Satina and Blakeslee (1941) found that the primordium is formed laterally from a superficial layer of small cells. The internode is probably differentiated across the lower limit of the dome and is therefore probably formed to a considerable extent from the large cells of which the core of the dome is composed.

After the unit is differentiated the cells in the two components differ not only with respect to division but also with respect to expansion. In the primordium the average volume increases during 7 plastochrons from about $2.3 \times 10^{-7} \text{ mm.}^3$ to about $8 \times 10^{-7} \text{ mm.}^3$. This increase, on the basis of the interpretation outlined above, suggests about a threefold increase during each cell generation which occupies something less than 4 days. As indicated above, the calculated values for average volumes in the internodes are subject to considerable error. Nevertheless, they suggest that there is little change in average volume as the internode ages. This suggests that during each cell generation which occupies about 5 days the volume only doubles.

The conclusions with respect to division and expansion in the internode may be considered in relation to the data of Table VI which show the length and breadth of the dome and of the successive internodes. These values have been obtained by microscopic measurement with a calibrated micrometer eyepiece. The length represents the distance between the upper and lower limits of the dome or internode, and the breadth the distance across the base of the structure at its widest point.

TABLE VI

Lengths and breadths (mm.) of the dome (D) and of the successive internodes

Fragment	Length	Breadth
D	0.08	0.19
1	0.04	0.28
2	0.06	0.32
3	0.04	0.37
4	0.05	0.41
5	0.03	0.48
6	0.04	0.55
7	0.06	0.62

Clearly as the internode ages, while there is probably little or no change in the length there is certainly a considerable increase in lateral extension. It has been shown above that during the course of growth there is probably little or no change in average cell volume, and the increase in breadth must therefore be due almost entirely to divisions occurring in the transverse plane. The data suggest that the increase in volume in the internode must be

attributed to divisions in which the long axis of the spindle is at right angles to the longitudinal axis of the stem. Cells are produced in the lateral plane and this leads to the lateral expansion of the internode without a corresponding change in the longitudinal direction.

In the previous section it was shown that both the volumes and the numbers of cells of the primordia and of the growth units increase exponentially with increasing plastochron age. The significance of this cannot be determined from the present series of data. In a situation in which the duration of the plastochron is constant and in which the rates of division and the relative rates of volume increase are constant then exponential series such as those recorded might be expected. At the same time it may be noted that in a system in which the ratio between the volume or the numbers of cells in the dome and the first growth unit or primordium remain constant, and in which the relative rates of volume or cell number increase during the plastochron are constant throughout the whole system of dome and several primordia then an exponential series is also obtained. This aspect of the situation is being further investigated.

ACKNOWLEDGEMENT

We wish to express our thanks for the valuable advice given to one of us (R. B.) by Professor R. H. Wetmore of Harvard University.

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An Analysis of the Influence of Plant Density on the Growth of *Vicia faba*

I. THE INFLUENCE OF DENSITY ON THE PATTERN OF DEVELOPMENT

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Received 14 August 1955

SUMMARY

The competitive effects of varying the density on the development of *Vicia faba* have been studied in a series of multifactorial experiments where the spacing both between and within rows was simultaneously altered. Over densities ranging from 11 to 67 plants per metre² seed production on an area basis tended to be maximal at 35-45 plants per metre² in the winter type and at the highest density for the spring type. As the density increased the number of pods per plant and the extent of branching fell progressively but there was no appreciable change in either seed size or the number of seeds per pod: thus seed production was solely governed by the number of mature pods formed. The number of flowers per plant was more dependent on the number of inflorescences than on the number of flowers per inflorescence. Increasing the population diminished the number of nodes bearing inflorescences particularly in the upper part of the shoot, while the size of the inflorescence was decreased to a lesser extent. The number of flowers forming mature pods was very small (9-14 per cent.). At the top of the shoot the flowers were infertile, while above the middle node most of the pods formed were shed while still immature. Thus the primary effect of increasing density was to depress the number of nodes on the lower half of the stems which produced mature pods. By the time the flowering stage was reached plant height was already correlated with density due more to a change in internode length than an alteration in the number of differentiated nodes; at maturity the differences in height were smaller due to the greater number of nodes in the widely spaced plants. For a given density, alterations in the distance between rows had little influence on development. The possible physiological factors responsible for the changing pattern of development brought about by varying density are discussed.

INTRODUCTION

IN a plant community any final analysis of the nature of the competition must involve a study of the interrelationships between the growth of the individuals and the growth of the whole population. For a society it has long been established, firstly, that one of the major factors delimiting the amount of growth per unit area is the density of the stand and, secondly, that the optimal range of density for maximal seasonal growth varies with the edaphic and environmental conditions. During the last half-century agronomists have assembled a vast array of facts linking the initial density with the final yield of a variety of crops but singularly little attention has been paid to the critical analysis of

Journ. of Experimental Botany, Vol. 7, No. 20, pp. 147-65, June 1956.

the interrelationships between density and the developmental changes that take place between sowing and maturity. Balls and Holton (1915) and Balls (1917) and Engledow and Wadham (1924) and Engledow (1925) were among the first to analyse critically the morphological components contributing to seed production in *Gossypium* and *Triticum sativum* respectively and both sets of workers made detailed studies of the morphological and ontogenetical changes induced in individual plants by varying the population. These investigations were followed by those of Clements, Weaver, and Hanson (1929) on *T. sativum* and *Helianthus annuus*, who concentrated their interest on the influence of spacing on the development of the individuals and did not attempt to assess the total growth of the several populations on the basis of unit area. Since that time and with the advances in the design of multifactorial experiments and their statistical interpretation basic studies of competition in which varying density has been combined factorially with other variables have been agronomical rather than ecological and perhaps the most intensive and extensive of these were initiated by Gregory, Crowther, and Lambert (1932) and continued by Crowther on the factors controlling the development and yield of *Gossypium* in Egypt and the Sudan. A feature of these investigations was the use of the concepts of growth analysis in the interpretation of the detailed records of multifactorial experiments, which sought to integrate the plastic responses of *Gossypium* to changes in density, varying water and nitrogen supply and attack by insect pests (e.g. Crowther, 1934 and 1936; Crowther, Tomforde, and Mahmoud, 1936).

Such studies are basic to a proper understanding of the principles of agronomy. It is, however, not always appreciated that in the absence of comparable ecological investigations such findings must serve as a critical basis for the elucidation of the nature of the competition within all plant communities.

In the present investigation an analytical approach has been adopted in a study of the factors controlling the growth and development of *Vicia faba* in the field. This paper relates to the plastic responses which are brought about by a change in density, while a second paper will be more concerned with an experimental analysis of the factors responsible for the induced variations in development.

Although for many centuries both spring- and autumn-sown types of *V. faba* have been important crops in Europe, surprisingly little research has been carried out in recent years on their agronomic requirements or the physiological factors determining the level of productivity. The long term experiments at Rothamsted (Hall, 1917) indicated that there was a considerable response to additional potassium while Oldershaw (1941, 1943) reported that the gains in yield due to potassium, particularly in the presence of phosphate, were greater than those of clover, wheat, barley, or mangolds. Boyd (1946), reviewing the results of short-term experiments at Rothamsted and elsewhere, concluded that 'beans stand in high need of potash', but nevertheless in two out of the six years there were no significant effects.

In Germany Bruns (1935) claimed that although the time of flowering and the course of pod formation are not affected by an increase in potassium status yet seed yield is considerably augmented. He postulated that potassium uptake is maximal during the vegetative phase with a secondary peak during the period of pod formation. Konold (1940) was the first to investigate the developmental factors which govern productivity and by a sub-sampling technique he estimated the values of three 'yield components', namely, plant density, the mean number of pods per plant, and mean pod weight. The main conclusions reached were that in general the production of seed per unit area increases with density, but that on a plant basis the number of pods and the yield are inversely correlated with density. He also concluded that for a given density alteration of row width had a negligible effect on yield. It should, however, be pointed out that alterations of density and row width were not simultaneously compared in multifactorial experiments.

Between 1947 and 1949 Soper (1952) carried out a survey of bean crops in the vicinity of Oxford and observations on plant development confirmed Konold's finding that pod production per plant is particularly sensitive to changes in density. At the same time it was observed that of the total number of flowers produced only a small proportion eventually became mature pods.

On *a priori* grounds it would be anticipated that *V. faba* should exhibit a high degree of plasticity to changes in the environment. Since the inflorescences are axillary the total number of flowers per stem will be dependent on the number of flower-bearing nodes and this in turn must depend on the length of the meristematic period during which new nodes are laid down. The extent of this development during the growing period is inevitably linked with internal physiological factors, which are in part dependent on the edaphic and environmental factors. Moreover, the size of the inflorescence may vary from one to ten or twelve flowers (Muratova, 1931) and appears to be determined in some measure by the position on the plant. Likewise, both the number of seeds developing in the individual pods and the ultimate size of the seed may be affected by both internal and external factors. In the autumn-sown strain, from one to seven lateral branches similar in form to the main stem may be produced at the basal nodes and in the axils of the cotyledons, and from field observation it would seem that lateral branching is linked with density.

Konold considered yield level simply in terms of only two variables—pods per plant and pod weight and it was thought that a greater knowledge of the plastic characteristics might be obtained by a further subdivision of the components into: (1) the number of stems bearing pods, (2) the mean number of pods on each stem, (3) the mean number of seeds in individual pods, (4) the mean size of individual seeds. In addition, in the later experiments counts were also made of the number of flowers produced at each node, together with records of the proportions of pods which either reached maturity or were abscinded at an earlier phase of development.

Furthermore, since Konold had investigated the effects of density and

row width in separate experiments multifactorial experiments including both these variables were undertaken.

EXPERIMENTAL METHODS

It is only in the last few years that any genetic selection of varieties within *V. faba* has been attempted; in consequence, the experimental material available was of a very heterogeneous nature. Initially, a local strain of autumn-sown type was employed but subsequently a more uniform strain was found in the neighbourhood of Rugby and this seed was used for the autumn-sown experiments in both 1950 and 1951. Seed from a Huntingdon strain was sown in the spring-sown trials.

The site of the 1949 trial was at Sandford-on-Thames where the area was a piece of rough grassland which was cultivated in the summer prior to sowing the seed in the autumn. The soil type was somewhat variable, consisting of Thames gravel overlying a mosaic of Coral Wrag and Kimmeridge Clay. In 1950 the experiments were removed to the new University Field Station at Wytham, where the soil was colluvium overlying a gravel terrace. Since the land had been fallowed in the summer of 1950 the weed population was relatively low. In 1951-2 another level and open site at the Field Station was selected. The soil, derived from the Oxford Clay, but with a small dilution of river gravel, was somewhat heavy.

All experiments were of the random block design, mostly of a factorial type. In the trials laid down in the autumns of 1949 and 1950 and the spring of 1951 the replication was two or, more usually, fourfold. Since it was found that the variability between replicates was high, subsequently the degree of replication was increased to six or sevenfold.

The main factor determining plot size was the labour demands for sowing, harvesting, and adequate sampling, but in the initial experiment there was also a limitation on the space available, and it was necessary to use plots of 0.008 hectares. This size proved very satisfactory from the viewpoint of labour requirements at harvest and the data obtained were sufficiently uniform to justify the continued use of this size in further trials. All the experiments received a basal dressing in the seed bed of 8 cwts. of basic slag and 1 cwt. of potassium chloride per acre.

Since these investigations were primarily concerned with a study of the effects of varying density on growth and development, to ensure the desired precision of spacing the seed was dibbled in by hand. Every effort was made to eliminate weeds at an early stage of growth and, whenever hand hoeing was demanded in any particular plot, the whole experiment was also hoed so as to obviate differential effects. Hoeing ceased when the plants reached a size where damage was liable to occur if further operations were carried out.

Periodic counts were made from flowering to maturity so that continuous records of flowering and the extent of pod formation could be obtained and even on the smallest experiment continuous observation on 480 plants was demanded. At harvest time plants from the outer rows of each plot were

discarded. It was also observed that there was an 'end of the row' effect, and plants within 30 cm. of the end were discarded.

Plots were harvested by hand pulling and the plot samples air dried in the field. In 1950 threshing was by means of a Shearer hand-operated pegdrum, but in subsequent years a Swedish machine, the 'Thermaenius', specially designed for plot work was substituted.

In 1950 the following sampling procedure was adopted. Thirty stems from each plot were randomly selected at harvest time and the total length and total number of nodes per stem, the height (height of first pod) and the node at which the first pod appeared (node of first pod), and the number of pods per stem were all recorded. Pods were stripped from the stem when counted and placed in a strong paper bag for further sampling. In 1951 the technique was modified so that the number of plants sampled per plot was related to the total population. For example, where the densities were 11, 22, 44, and 66 plants per square metre every second, third, fourth, and fifth plant respectively was taken. From each plot sample of mature pods some fifty pods were selected and the mean number of seeds per pod and the mean seed weight determined.

From all these data it is possible to calculate the theoretical plot yield and it is of interest to note that in the principal density experiments of 1951 and 1952 the mean percentage deviation of the calculated from the observed level of yield ranged from -2.3 to -4.7 per cent.

EXPERIMENTAL RESULTS

Autumn-sown varieties

The first and second trials in the series of spacing experiments were identical in design, but in the third the treatments were modified in the light of previous results. Experiments 1 and 2 were based on a combination of four sowing rates, namely, 11, 22, 44, and 66 seeds per square metre and three different distances between rows—namely, 18, 36, and 54 cm. Thus within the resulting twelve treatments the spacing was such that not only did the number of individuals vary per unit area, but that within areas of equal size the plants were distributed in a changing pattern. In Experiment 3 the 18 cm. row spacing was discarded, leaving a total of eight treatments with six rather than fourfold replication.

The survey by Soper (1952) demonstrated that a high mortality during the winter may arise not only from the depredation of rooks and mice, but also from a type of 'winter killing' which has not, as yet, been fully investigated. It was therefore decided to include at the start of each spacing experiment an additional 'nursery' area from which plants could be transplanted to those plots which had suffered winter losses. Mortality in the winter of 1949 was primarily caused by mice which continued to destroy the slowly germinating seed throughout December, in spite of intensive trapping. In 1950 reduction in plant density was chiefly due to winter killing—typified by a blackening

of the epicotyl, followed by withering of the main stem. Rooks also caused some damage in spite of the efforts made to protect the experimental area by stretching strong black cotton at approximately 1 metre above the ground. The autumn and winter of 1951 favoured the germination and early growth of the plants and mortality was so low that transplanting was unnecessary. At the lower plant densities in Experiments 1 and 2, where the plants were widely spaced, it was possible to replace almost every missing plant. At the higher densities, however, where the plants were closely spaced within the row, it was impossible to maintain the planned densities without unduly disturbing established plants; in such cases only the obvious gaps were filled.

It is realized that the method used in maintaining plant density is open to the criticism that the ultimate development of a transplanted plant may differ from that of an undisturbed plant. Every effort was made to reduce the check in growth when the operation was carried out. The plants were moved as soon as possible in the spring, when they were still at an early stage of development, and so relatively easy to handle; they were transferred rapidly from the nursery bed to the plots, and any plant with a damaged tap root or not surrounded by a 'ball' of soil was rejected. This method appeared to be successful in that no check in growth was observed.

TABLE I

Experiments 1, 2, and 3. The Interrelationship between Plant Density and Seed Production in Autumn-sown Crops

Density		Plants/ metre ²	Seed weight (g.)	No. of seeds per pod	No. of pods per stem	No. of stems per plant
Experiment 1						
Very low	. . .	10.7	0.60	2.86	6.47	2.66
Low	. . .	18.9	0.61	2.94	5.43	2.09
Medium	. . .	29.0	0.65	2.82	4.36	1.71
High	. . .	41.9	0.65	2.78	3.76	1.36
Sig. diff. (P=0.05)			0.04	0.10	0.37	0.11
Experiment 2						
Very low	. . .	11.3	0.72	2.70	6.06	2.41
Low	. . .	21.9	0.74	2.73	4.23	2.15
Medium	. . .	39.0	0.76	2.69	3.06	1.93
High	. . .	55.3	0.74	2.60	2.70	1.73
Sig. diff.	. . .		0.03	N.S.	0.34	0.09
Experiment 3						
Very low	. . .	11.2	0.51	3.04	6.65	2.50
Low	. . .	21.0	0.50	3.02	5.34	2.05
Medium	. . .	43.3	0.53	2.88	4.06	1.57
High	. . .	64.8	0.52	2.85	3.52	1.29
Sig. diff.	. . .		N.S.	0.11	0.55	0.14

Since seed production is dependent on the integrated effects of treatments and environment on the previous growth and development, the interrelationships between density and seed production, both on a unit area and plant basis, have been considered first. These considerations have been followed by an analysis of the contributions made by the different components to seed production, and finally the resultant effects of the early growth of the developing plant has been taken into account.

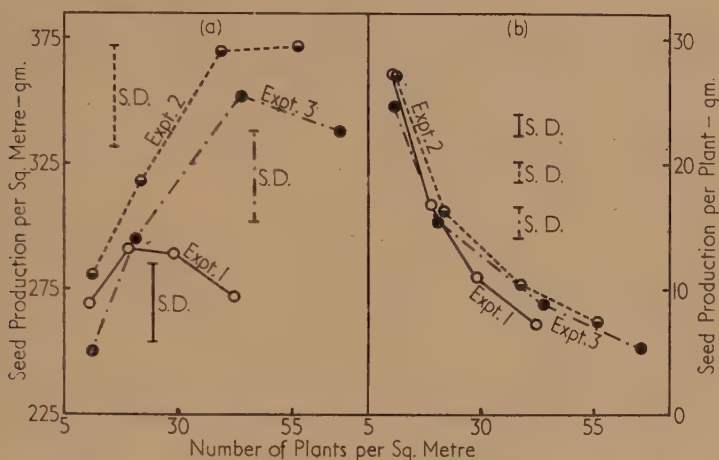


FIG. 1. The influence of varying density on seed production (a) per metre² and (b) per plant.

The interrelationship between plant density and seed production. When the data are examined on the basis of productivity per unit area, it is seen from Fig. 1 (a) that with increasing density the yield of seed rises to a maximum, after which there is no significant change. This trend is common to all experiments, although in Experiment 1 there is no significant difference between densities. There is some overall indication that at plant densities greater than the medium density yield may decrease. From Fig. 1 (b) it is evident that seed production per plant falls sharply as the density increases. In Experiment 1, at plant densities greater than the low density, the increase in plant numbers fails to compensate for this fall; in the other experiments up to the medium densities the decrease in production per plant is more than offset by the increase in plant numbers. When these same results are examined to determine the effects of spatial arrangement at any given density it is found, somewhat surprisingly, that there are no significant effects; indeed, the means for row width are in very close agreement.

The interrelationship between density and plant development. In Experiments 1 and 2 the individual seed weight was affected by density (Table I) but in none of the experiments was there any significant influence of spatial arrangement. Although with rising density there are definite changes in seed weight in two out of the three seasons, they are small compared to the fluctuations from season to season.

Within the limits of the experiments, neither changes in the plant population nor the spatial arrangement appear to have a pronounced effect on the number of seeds per pod. Moreover, variations from season to season are small (Table I). The greatest variation attributable to changes in density occurs in Experiment 3 where the number of seeds at the highest density is 3.4 per cent. lower than the mean for the experiment (2.95 seeds per pod). Seasonal variation between the mean values is never greater than 10 per cent.

Within the range of the densities covered by the experiments the number of pods per stem falls consistently and rapidly as the density of the population increases (Table 1). Even though the data indicate that this yield component is highly dependent on the number of plants per unit area, the variations in spatial arrangement have no significant effect on pod production.

With increasing density there is a highly significant reduction in the total number of stems produced by each plant (Table I). The extent of this reduction, however, varies between experiments. In Experiment 1 the reduction is very striking, whereas in Experiment 2 where the range of density is wider the suppression of branching is much less marked. Although statistical analysis indicates that stem production is affected by spatial arrangement in Experiments 2 and 3, these effects are small when compared with those due to changes in population. At the equivalent densities plants in rows 35 cm. apart produce some 4.6–5.2 per cent. more stems than plants at a row width of 44 cm.

TABLE II

Experiments 2 and 3. The Interrelationship between Density and the Percentage of Stems Lacking Pods

Plant density	Percentage of stems without pods	
	Experiment 2	Experiment 3
Very low	5.9	6.0
Low	6.5	5.9
Medium	11.2	9.6
High	14.3	17.4

When the number of stems per plant is further analysed for the effects on the number of stems with and without pods, then it is found that the number of podless stems increases with density (Table II) and thus the number of productive stems per plant diminishes even more rapidly than is indicated in Table I. In the course of the experiments it was observed that although the main production of lateral branches occurred in the early spring, branching did not entirely stop until the plants were in the flowering state and at the lowest densities, irrespective of spatial arrangements, all but the last stems to be formed were capable of producing pods. At the very low light intensity prevailing within the higher densities these late stems, though producing flowers, seldom produced pods.

The interrelationships between plant density, flower production, and pod formation. In Experiment 1 by mid-May (1950) there was already a very marked difference in the height of plants from plot to plot, and the length of

each main stem and the number of nodes per stem were measured in the early flowering phase. Whilst these data show that there is a highly significant increase in length of stem with increasing density (Table III) there is no indication that the total number of nodes per plant varies with density. Thus increases in the length of stems are entirely due to increases in internode length.

TABLE III

Experiment 1. The Interrelationship between Plant Density and the Development up to the Early Flowering Phase

Density	Plants/ metre ²	Length of stem (cm.)	Height of 1st inflores- cence (cm.)	No. of nodes on main axis	Mean inter- node length (cm.)
Very low	10.7	83.7	31.2	11.6	7.2
Low	18.9	95.0	36.5	11.7	8.1
Medium	29.0	109.3	43.5	11.6	9.4
High	41.9	115.5	51.8	12.0	9.6
Sig. diff.		4.9	5.4	N.S.	

Although just significant at the 0.05 level, spatial arrangement accounts for only a difference of 5.0 cm. in height between the 18 cm. and 54 cm. row widths, whereas a variation of over 20 cm. occurs between the lowest and highest densities. There is an evident correlation between the length of the stem and the height of the first inflorescence (Table III). The results show that this linkage is the consequence of internode elongation.

Since in Experiment 1 it was observed that a large proportion of the flowers failed to develop into mature pods, in Experiment 2 flower counts were made to obtain an accurate measure of the losses. The total number of nodes at which at least one flower was formed (number of flowering nodes) was also recorded and, as in Experiment 1, records were made of the position of the first inflorescence (node of first inflorescence). The data show that flower production per stem decreases rapidly as the density increases (Table IV). At the highest density flower production is 44.7 per cent. less than at the lowest density studied. The chief factor responsible for this reduction is seemingly the variation in the number of nodes bearing inflorescences rather than the number of flowers per node. In parenthesis, it is recorded that variations in spatial arrangement, at any density, have no statistically significant effects on the number of either flowers produced or nodes bearing inflorescences.

Like the number of nodes bearing inflorescences the number of nodes bearing pods (pod bearing nodes) diminishes as density increases (Table IV), but the means of 9.82 and 3.37 nodes are markedly different. This reduction in the number of 'fertile nodes' is primarily due to the abscission of the inflorescences at the upper nodes of the plant—see Fig. 2—but in some seasons this complete loss of flowers also occurs at the lower nodes (Table V). There is

TABLE IV

Experiment 2. The Interrelationship between Plant Density and the Pattern of Flower and Pod Production in an Autumn-sown Crop

	Plants/ metre ²	Number of flowers/ plant	Flowers per stem	No. of flowers per node	No. of flowering nodes	No. of pod- bear- ing nodes	Percent- age of flowers forming mature pods
Plant density							
Very low	11.3	138.0	49.4	4.03	12.25	4.78	14.2
Low	21.9	97.4	41.4	3.87	10.59	4.70	11.5
Medium	39.0	72.7	33.0	3.70	8.79	2.68	9.3
High	55.3	55.8	27.5	3.55	7.70	2.32	9.4
Sig. diff. ($P=0.05$)		4.7	4.0	0.35	0.50	0.37	0.9

some indication that a greater proportion of inflorescences fail to set seed at the higher densities. At the very low density 61.0 per cent. of the flowering nodes bear no pods whilst at the high density the figure rises to 70 per cent.

The results of Experiment 2 show that over the whole experiment only 11.1 per cent. of flowers developed into mature pods. The data suggest that, compared to pod production, flower production is less sensitive to changes in density. At the very low density 14.2 per cent. of the flowers become mature pods, whereas at the high density the percentage falls to 9.4 per cent. (Table IV).

Again it is recorded that the percentage of flowers forming mature pods is not significantly dependent on spatial arrangement.

When the main stems and lateral branches are considered separately (Fig. 2) the general pattern of flower and pod production is similar. Moreover, statistical analysis showed no significant difference in the percentage of flowers forming mature pods on main stems and branches. In consequence no distinction has been made between main stems and lateral branches in combining the results of Experiments 1, 2, and 3.

In both Experiment 1 and 2 the data indicate that the position of the first inflorescence remains relatively unaffected by changing density (Table V). The node at which the first pod occurs was recorded in each experiment and the results (see Table V) show that a change in density has a small effect since the first pods tend to be borne at a higher node as the density increases.

In contrast to the node of the first inflorescence, the node of the first pod varies markedly from season to season. In Experiment 1 the mean position of the first inflorescence was at 5.60 nodes whilst the position of the first pod was 11.56 nodes, the flowers at the five intervening nodes having failed to develop. In Experiment 2 the means of the first inflorescence and the first pod varied by only one node. These observations are in accord with those made by Soper (1952) who reports that, under field conditions, even though the earliest flowers usually occurred at about the sixth node, the node of first pod varies from season to season.

The total number of nodes on the stem decreases significantly with increasing density in each of the three experiments, while in contrast the height

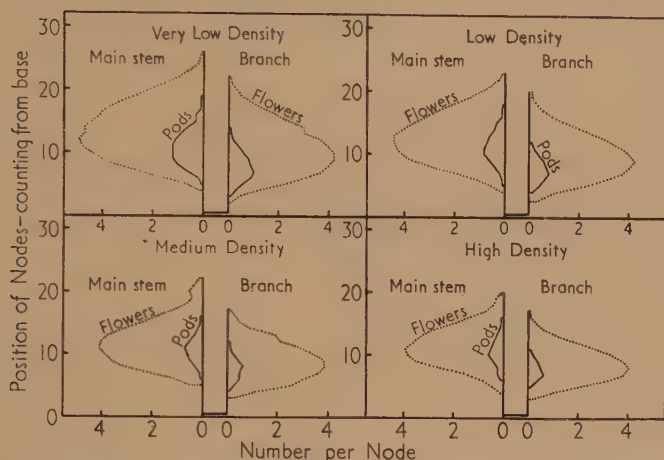


FIG. 2. *Expt. 2.* The effects of varying density (11.3, 21.9, 39.0, 55.3 plants/metre²) on the nodal distribution of the number of (i) flowers, (ii) mature pods.

increases—Table V. Thus, over the range of densities involved the internode length becomes progressively greater as the density of plant population increases.

In the spacing experiment with the spring-sown type the final densities were 10.9, 22.4, 45.9, and 67.2 plants per square metre. The criteria of yield, growth, and development recorded were the same as those in the previous experiments, but in this instance a distinction was made between the abscission of flowers and small developing pods, an intermediate count being carried out when the pods were 10–15 mm. in length. Such pods will be termed immature pods.

The interrelationship between plant density and seed production. From Table VI it is evident that seed production per unit area increases with density and that the increments at the successively higher densities are similar over the whole range. On the other hand the seed production per plant decreases sharply as density increases. The mean seed weight remains constant over the whole range of densities, but there is a small but significant decrease in the number of seeds per pod with increase in density. As in the autumn sown type the number of pods on each stem decreases with increase in density: between the very low and the high density there is a 56.8 per cent. reduction in the number of pods produced. It is to be noted that even at the very low

TABLE V

Experiments 1, 2, and 3. The Interrelationship between Plant Density and Selected Morphological Characters

	Plants/ metre ²	Node of first inflor- escence	Node of first pod	Height of first pod (cm.)	Total plant height (cm.)	Total no. of nodes per stem	Mean inter- node length (cm.)
Experiment 1.							
Very low . . .	10.7	5.50	11.13	70.8	151.5	24.8	6.11
Low . . .	18.9	5.42	11.67	82.6	156.4	24.1	6.49
Medium . . .	29.0	5.48	11.54	91.8	156.8	22.7	6.92
High . . .	41.9	6.00	11.88	96.5	154.6	22.0	7.05
Sig. diff. . . (P=0.05)		N.S.	N.S.	4.2	N.S.	0.7	0.13
Experiment 2.							
Very low . . .	11.3	5.92	6.27	24.9	89.6	20.3	4.41
Low . . .	21.9	6.34	6.88	33.7	95.5	18.9	5.05
Medium . . .	39.0	6.27	7.57	45.0	100.3	18.4	5.45
High . . .	55.3	6.64	7.93	49.9	99.8	17.6	5.68
Sig. diff. . .		0.48	0.34	2.3	3.8	0.7	0.08
Experiment 3.							
Very low . . .	11.2	—	7.11	38.0	124.5	23.7	5.21
Low . . .	21.0	—	8.09	50.6	129.3	22.0	5.92
Medium . . .	43.3	—	9.02	66.8	133.0	21.0	6.34
High . . .	64.8	—	9.23	72.7	128.2	19.5	6.55
Sig. diff. . .			0.40	2.5	4.5	1.2	0.11

TABLE VI

Experiment 4. The Interrelationship between Density and Seed Production

	Density				Sig. diff. (P = 0.05)
	Very low	Low	Medium (plants/metre ²)	High	
Calculated seed yield per metre ² (g.) . . .	10.9	22.4	45.8	67.2	
Seed yield per plant (g.) . . .	291	305	331	355	26
Number of stems per plant	24.8	13.6	7.3	5.3	1.3
Number of pods per stem	1.88	1.23	1.01	1.00	0.05
Number of seeds per pod	7.48	6.47	4.34	3.23	0.33
Mean seed weight (g.)	2.86	2.72	2.57	2.47	0.15
	0.69	0.69	0.69	0.69	

density few branches are formed, while the production of laterals is completely inhibited at the high density.

The interrelationships between plant density, flower production, and pod formation. The effect of increased density on flower production is shown in Table

VII. As the density increases the number of flowers on each stem falls markedly: comparing the lowest and high densities the percentage reduction is 62.8. The pattern of immature pod production is similar to that of flower production. As with the autumn type, when the density is increased, the reduction in the number of 'flowering nodes', and later in the number of nodes bearing pods, seriously limits first flower and then pod production per plant (Table VII).

TABLE VII

Experiment 4. The Interrelationship between Density and the Pattern of Flower and Pod Production in a Spring-sown Crop

	Density (plants/m. ²)				
	Very low	Low	Medium	High	Sig. diff.
	10.9	22.4	45.8	67.2	
Number of flowers/plant	121.7	64.7	36.8	22.7	3.5
Number of flowers/stem	60.6	52.0	36.2	22.6	5.1
Number of immature pods per stem	25.3	23.0	15.2	10.4	2.4
Percentage of flowers forming immature pods	41.7	44.5	42.2	46.4	N.S.
Percentage of flowers forming mature pods	12.9	13.1	12.3	14.4	N.S.
Number of 'flowering nodes'	10.72	9.79	7.86	6.14	0.63
Number of flowers/node	5.64	5.30	4.59	3.67	0.14
Number of nodes bearing immature pods	8.85	8.35	6.90	5.15	0.77
Number of nodes bearing mature pods	4.01	3.58	3.61	2.38	0.29

It was observed that there was a complete loss of flowers at some nodes. These losses were not confined to the flowering stage and the proportion of 'barren nodes' increased as the plants continued to develop. From Table VII it is evident that these losses occurred irrespective of the density. Thus, the ratio of 'flowering nodes' to nodes bearing mature pods was approximately 5 : 2 and remained relatively unaffected by density.

Since the positions of the first inflorescence and the first mature pod are the same (Table VIII) it follows that the reduction in the number of fertile nodes must be linked with abscission at the upper nodes. That such a loss, first of flowers and then of immature pods, occurs in the top half of the stems is evident from Fig. 3. This figure also brings out the extent to which density influences the position and number of nodes bearing mature pods. Both Fig. 3 and statistical analysis indicate that between the main stem and lateral branches there is a small but significant difference in the percentage of flowers forming pods. On the other hand the patterns of flower, immature pod, and mature pod production were similar in both the main stem and lateral branches. It is to be noted that over all densities only 13.2 per cent. of the flowers produced mature pods, a figure corresponding very closely with the value of 11.1 per cent.

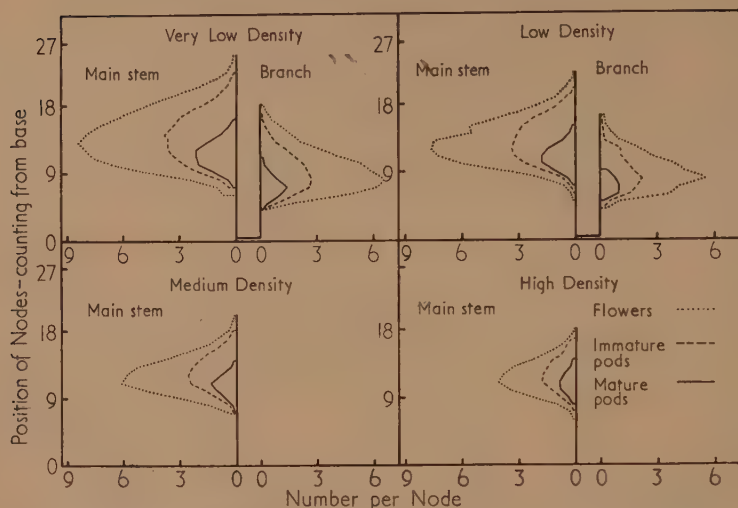


FIG. 3. *Expt. 4.* The effects of varying density (10.9, 22.4, 45.8, 67.2 plants/metre²) on the nodal distribution of the number of (i) flowers, (ii) immature pods, and (iii) mature pods.

observed in the winter-sown experiments. However, there is a marked difference in the two types in that in the spring-sown populations the percentage of flowers forming mature pods does not alter significantly with changes in density.

TABLE VIII

Experiment 4. The Interrelationship between Plant Density and some Selected Morphological Characters

	Density				Sig. diff.
	Very low	Low	Medium (plants/metre ²)	High	
	10.9	22.4	45.8	67.2	
Node of 1st inflorescence	7.15	8.37	9.27	9.58	0.41
Node of 1st immature pod	7.25	8.49	9.30	9.63	0.39
Node of 1st mature pod	7.64	8.76	9.43	9.80	0.39
Height of 1st mature pod (cm.)	48.2	57.4	70.4	76.3	2.4
Height of mature plant (cm.)	101.6	104.0	106.9	106.0	3.9
Total nodes per stem	20.9	21.5	19.8	18.4	0.51
Mean internode length (cm.)	4.9	4.8	5.4	5.8	

The interrelated nodal positions of the first inflorescence, immature and mature pod remain relatively unaffected by changes in plant density (Table VIII), but as density increases the first inflorescence is borne at a higher node. The significant increase noted in the height of the first pod (Table VIII)

may be attributed to the combination of an increase in the number of internodes and a gain in the mean internode length at the higher densities, since though plant height does not increase significantly with density there are significantly fewer nodes.

DISCUSSION

The present experiments have established that from the viewpoint of seed production by both autumn- and spring-sown types of *V. faba* the population is largely self-balancing over a range of a five to sixfold variation in density. In other words, the plastic response of the individual plants is such that a change in numbers is inversely correlated with the amount of seed produced per plant. At least for the autumn-sown type there is some indication that the correlation is not linear over the whole density range, since in Fig. 1a the trends for the three experiments suggest that the production of seeds per unit area is maximal when the density is about 35–45 plants per square metre. For the spring-sown type, although the results of the single trial require confirmation, the indications are that the optimal density exceeds 67 plants per square metre (Table VI). In parenthesis, Soper (1952) has concluded that compared to the autumn-sown type a higher seed rate is demanded.

From the developmental data it is possible to analyse with precision the nature of the plastic responses induced by changing the density and thereby to throw some light on the intensity and the nature of the competition between plants. For many of the characteristics the reactions of the spring- and autumn-sown types are the same. For example, the linkage between density and seed production per plant is largely delimited by the number of mature pods produced and not by either the weight of the individual seeds or the number of seeds per pod (Tables I and VI). The pod production per plant can again be split into two further components, the degree of branching and the number of pods per stem. For both types branching decreases as the density increases (Tables I and VI) while the autumn type is more prone to branch. On the other hand, the rate of fall in pod production per stem with rising density is approximately logarithmic and is of the same order for both types (Tables I and VI).

The production of ripe pods is clearly dependent on the integrated effects of the internal physiological factors and the external environmental conditions on the development which has gone forward from the early vegetative phase. Thus, the main stem, together with the axillary branches in their final form provide the framework for the production of flowers which in turn is linked with the final pod production. Since the inflorescences are axillary the flower potential is dependent on the mean number of nodes at which inflorescences can be produced and the mean number of flowers per node. From Tables V, VII, and VIII it is evident that flower production is not directly proportional to the number of fully differentiated nodes per plant. For instance, in experiment 2 between the highest and lowest densities the percentage decrease in flower number is 60, while the corresponding reduction in the total number of nodes is only 38. Such differences arise from three further responses.

Although the number of nodes per stem is not greatly dependent on density (Tables V and VIII) the number of nodes at which flowering takes place on a stem is greatly affected. In both the spring and autumn types the fall in the flower-bearing nodes over the limits of density is of the same order—37 per cent. in Experiment 2 and 43 per cent. in Experiment 4 (Tables IV and VII). The remaining contributory factor is the number of flowers per node. Here there are differences between Experiments 2 and 4. For a fivefold increase in density (Experiment 2) the mean falls by 19 per cent., while for a sixfold change in density (Experiment 4) the corresponding decrement is 34 per cent. (Tables IV and VII).

The final linkage between flower production and the reproductive capacity of the plant is the ratio of mature pods to total flowers. Once more, there seems to be some difference between the spring and autumn types. In Experiment 2 the percentage number of flowers which develop into ripe pods falls with density (Table IV), while in Experiment 4 the variation is not statistically significant. For the spring type in the progression from flowers to ripe pods more than half the flowers fail to produce immature pods, while in turn more than half the immature pods fail to reach maturity (Table VII).

It is not proposed to discuss in detail the nature of the physiological and environmental factors which govern the interrelationships between density and the changing plastic pattern, since such a discussion is best left for a further paper, where additional experiments aimed at assessing the nature of the competition are described. Nevertheless, there are a number of relevant points which should be made.

By the time the initial flowering phase is reached in the autumn-sown type the closest spaced plants are the tallest, due not to a change in internode number on the main stem but to an increase in mean internode length (Table III). Now Crowther (1934) in his studies of the effects of varying nitrogen and water-supply on the development of *Gossypium* has shown that while internode number on the main axis is linked with the nitrogen level the mean length is positively correlated with the availability of water. In addition, other workers have shown for many species that branching or tillering, at least in the early vegetative phase, can be limited by the supply of nitrogen substrates. On such bases it could be inferred that the formation of the initial branches is associated with a varying level of nitrogen. It could also be advanced that at least up to the full development of the first inflorescence the number of nodes on the main stem is not so dependent since these nodes have already been laid down in the early seedling stage before competition for nitrogen comes into operation.

The fact that internode length is greatest at the highest density suggests that some other factor than availability of water must come into play. It has long been established that shading within limits increases internode expansion and it would therefore seem that with increasing density competition for light is already in progress by the time the first flowers open.

In the autumn-sown type the failure of varying density to affect the number

of nodes on the main axis up to the end of the vegetative phase is matched by the relatively small influence of density on the nodal position of the first inflorescence. In this connexion Grainger (1948) claims that flower initials are already laid down before the shoot of *V. faba* emerges from the soil. Thus, at a stage of ontogeny up to the end of the winter it would not be expected that the differential effects of density would play a large part in determining the position of the first inflorescence or the number of nodes. The position of the spring-sown type seems rather different since density has an appreciable effect on the node of the first inflorescence. This disparity between types suggests either that the initial inflorescences are laid down somewhat later, or that in the absence of an overwintering period competition starts at an earlier ontogenetic phase.

The growth of *V. faba*, like that of *Gossypium*, is indeterminate and it has already been pointed out that by the time maturity is reached the final number of differentiated nodes is inversely linked with density. From Table IV there is some indication that over a comparable range of densities the final number of nodes varies between experiments. For a given density the eventual number per plant will be associated with the amount of nitrogen available in the plant and the partition of the nitrogen between nodes, leaves, inflorescences, and developing pods. Moreover, the intensity of the internal competition for the nitrogenous resources will be dependent on both the stage of development and the amounts of nitrogen derived from the nodules or absorbed by the roots.

Although in leguminous plants much of the nitrogen may come from the nodules there is evidence at least for some species that the contribution resulting from uptake from the soil may be important. For example, it has been demonstrated that additions of inorganic nitrogen to the soil can increase the growth of peas (Woodman, 1944), soyabean (Norman, 1943, and Norman and Krampitz, 1945), and *Trifolium repens* (Blackman and Templeman, 1938), while Masfield (1952) has concluded that for a number of legumes the extent of nodulation and the interval between the formation and disintegration of the nodules varies widely in the field. Thus the postulate that with an increase in density of *V. faba* competition for nitrogen is accentuated is not incompatible with these findings. Moreover, the importance of the supply of external nitrogen may well vary with the phase of development. The effects of crowding in suppressing the initial branching could be explained on the basis that at this early stage nitrogen fixation has yet to reach maximal activity. At a later phase another factor may come into operation. The increased shading at the higher densities may affect both the efficiency of nitrogen fixation and the period during which the nodules remain active. The fact that a rise in density causes a greater depression of flower and pod production in the upper half of the stem is suggestive of a greater limitation of nitrogen towards the end of the flowering period. In this connexion Crowther (1936) and Crowther, Tomforde, and Mahmoud (1936) have demonstrated that in *Gossypium* widely spaced, as against closely spaced, plants produce a higher proportion of flowers and bolls in the middle and upper nodes and that the

effects of additional nitrogen in augmenting boll number is restricted to the upper half of the shoot.

While this discussion has emphasized the possible interrelationships between nitrogen supply and the plastic responses to changing density, it should also be stressed that competition for carbon substrates between the lower and upper parts of the plant must also be taken into account. It will be shown in the second paper that by the time the stage of full flowering is reached the self-shading at the high densities has reached a level where in the lower half of the shoot little active carbon fixation can be proceeding. In consequence, the requirements for carbohydrates in the developing pods at the lowest nodes will influence terminal growth and development.

In conclusion, this investigation has demonstrated how complex may be the phenotypic responses to the changing conditions arising from variations in the density of the plant population. So far comparable comprehensive data are only available for a few species and it is evident that before the principles of phenotypic response to varying degrees of competition can be put on a proper ecological basis studies must be made of a wide range of plants of different morphological types. The need for such extended studies is well illustrated by current research on *Papaver somniferum*. Although like *V. faba* the flowers are axillary and branching is restricted, the plastic responses to varying density are quite different. All the flowers that are formed produce capsules and the variation in seed production with density is dependent both on the number of flowers formed and the size of the capsule.

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Fat Metabolism in Germinating *Citrullus vulgaris*

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Received 24 August 1955

SUMMARY

A column chromatographic technique, enabling identification and quantitative estimation of fatty acids, has been employed to study fat metabolism in *Citrullus* during germination in the light. This plant is characterized by an unusually rapid disappearance of storage fat as the cotyledons expand and turn green. In spite of the high catabolic activity there is no evidence for accumulation of free fatty acids or short-chain fatty acids at this stage. Information on this point derived from acid value or saponification value of the oil is shown to be untrustworthy.

Citrullus seed fat contains the following percentages of acids: linoleic 70.6, oleic 7.2, palmitic 10.1, stearic 11.2, and arachidic 0.6, and careful analysis has also revealed small amounts of octadecatienoic acids, both conjugated and non-conjugated. All the major acids are broken down at rates proportionate to the quantities originally present, with the exception of oleic acid which is metabolized somewhat more rapidly.

'Linolenic' acid is synthesized in the expanding green cotyledons and the fatty acid composition of the latter, in the late germination stages, resembles that of a green leaf and is very different from that of the seed.

The results suggest a rapid removal of storage fat from the cotyledons and concomitant formation in small quantity of a typical leaf fat as the new photosynthetic function develops.

INTRODUCTION

BIOCHEMICAL changes during and following germination have, in the past, been investigated in a number of seeds which contain a high proportion of reserve fat. The latter may be stored either in the endosperm as in *Ricinus* and *Gossypium* or in the embryo itself—usually in the cotyledons. These may remain permanently below ground as in *Corylus*, or they may emerge, turn green, and function photosynthetically as in *Helianthus*, *Cucurbita*, and *Citrullus*.

In earlier investigations seedlings have usually been grown in darkness or in a CO₂-free atmosphere in the light and a correlation between fat loss and carbohydrate increase has been established in *Helianthus* (Miller, 1910; 1912), *Cucurbita* (Jegerow, 1906; Zeller, 1935; Weber, 1936; Heumann, 1944) and many other genera (*vide* von Ohlen, 1931; Murlin *et al.*, 1933; Houget, 1942, 1943; Johnston and Sell, 1944; Dupéron, 1954). The fi

metabolism of seedlings grown in the dark is known to be different from that of the same plants grown under normal conditions (Hée and Bayle, 1932; MacLachlan, 1936; and Van Fleet, 1950) and, in order therefore to study biochemical changes during the progress of the cotyledons from fat storage to photosynthetic function, the present investigation has been carried out on seedlings reared in the light.

Individual fatty acids were seldom estimated in the earlier work, the majority of conclusions being based on oil constants such as iodine, saponification, acid, and thiocyanogen values. Such determinations give seriously incomplete information, particularly when extraneous material forms a major fraction of the so-called lipid extract as happens in the later stages of germination. In order to obtain reliable experimental information a modern chromatographic technique for fatty-acid identification and determination has been employed in the present study. It is believed that this is the first example in which a complete fatty-acid analysis of a germinating seed has been made.

METHODS

Plant material. Dry seeds of *Citrullus vulgaris* var. Tom Watson (a gift from Ferry-Morse Seed Co., California) were soaked in tap water at 27° for 24 hours, then planted out in sand. The main batch of material on which analytical work was carried out was planted in March 1954, and seedlings were grown in daylight with additional fluorescent lighting between 6.30 a.m. and 6.30 p.m. The mean daily temperature was 31°, varying between 45° and 24° C. Duplicate random samples of 100 seeds each were taken at 0, 4, 6, 7, 9, 12, and 16 days from the start of the experiment. The testas were removed and each sample (except the first) was divided into cotyledons and 'rest of the plant'.

Fresh and dry weights. The material was washed free from sand, drained, blotted, and weighed for fresh weight. It was then dropped into boiling acetone, the solvent distilled, and the residue heated at 80°–85°, all operations being conducted in a stream of nitrogen. After grinding, drying was continued to a constant weight at this temperature under nitrogen.

Fat extraction. The dried material was continuously extracted for 6 hours with light petroleum (unless otherwise specified this refers to purified material b.p. 40°–60°) in a Soxhlet apparatus. The solvent was evaporated at low temperature under nitrogen, the extract weighed, dissolved in carbon tetrachloride, and stored under argon in sealed tubes until required for analysis.

Free fatty acids. After removal of solvent the acid value of the lipid extract was determined according to a standard method (Association of Official Agricultural Chemists, 1950, method 26.30). The extracts from the later stages were coloured, and titration (endpoint phenolphthalein, pH 9.4) necessitated use of a glass electrode. The resulting neutral solution was then partitioned between light petroleum and dilute sodium carbonate solution. The combined aqueous extracts were acidified with hydrochloric acid and the

free fatty acids extracted with light petroleum and washed with water. After evaporation of the solvent the residue was analysed chromatographically.¹

The neutral light petroleum extract was treated as follows.

Saponification of the neutral light petroleum extract. The solvent was removed and saponification carried out by a standard method (American Oil Chemists Society, 1953, Cd 3-25). Non-saponifiable material was removed by extraction with ethyl ether, washed, dried, the solvent evaporated, and weighed. The aqueous solution of soaps was acidified with hydrochloric acid and the fatty acids extracted with light petroleum, washed, and analysed chromatographically. This fraction is designated 'combined fatty acids'.

Reversed-phase chromatography of fatty acids

The analytical method employed has already been described in detail by Crombie, Comber, and Boatman (1954, 1955). It uses a reversed-phase partition technique in which the fatty acids are eluted from a column of silane-treated, water-repellent kieselguhr, loaded with liquid paraffin, by acetone-water mixtures. The method is applicable to estimation of saturated fatty acids varying in chain length from C_6 to C_{24} : shorter chain acids can be detected but not separated. The latter have not been encountered in the present work. The behaviour and estimation of a wide range of unsaturated fatty acids has been examined and for full details the above references should be consulted.

Ultra-violet spectrophotometry of fatty acids

Conjugated acids were determined by direct spectrophotometry either on the original oil or on the 'combined fatty acid' extract. The material was dissolved in spectroscopically pure absolute ethanol (B.D.H.) and corrections for background absorption applied (American Oil Chemists Society, 1953, Cd 7-48).

Linoleic and linolenic acids were estimated by ultra-violet light absorption after isomerization at 180° in the presence of potassium hydroxide (10 per cent.) dissolved in pure glycerol (Brice *et al.*, 1952), measurements again being made in ethanol.

A Hilger photoelectric 'Uvispek' was used for all the above determinations.

Carbohydrates (reducing sugars, sucrose, and starch). The residue after extraction with light petroleum was refluxed for 3 hours in 80 per cent. ethanol. The product was cooled and solid material separated by centrifugation and washed with 90 per cent. ethanol. Sucrose and reducing sugars were estimated on the aqueous alcoholic extract by the method of Van der Plank (1936). The residue was homogenized in water and the starch brought into colloidal solution by adding boiling water and maintaining at 100° for 20 minutes. The starch was hydrolysed by two successive 24-hour treatments

¹ Since preparation of the sample might have caused some loss of volatile free fatty acids, a special search was made at the stage of rapid fat breakdown using method Cd 5-40 of the American Oil Chemists Society, 1953.

with 0.1 per cent. α -amylase (L. Light & Co.) at pH 4.8. After filtration, hydrolysis was completed by heating the filtrate to 100° in N hydrochloric acid for 1 hour. The acid was neutralized with solid sodium carbonate and the reducing sugars formed estimated as above; the factor taken for conversion of glucose to starch was 0.90.

EXPERIMENTAL RESULTS

External appearance, fresh weight and dry weight. A brief description of the external appearance of the seedlings at different stages is given below in order to indicate their general growth. A full photographic record is given by Comber (1955). In the 4-day stage material radicles had emerged in a few seeds and at 6 days variation between individuals was more marked than at any other stage (some seedlings had partly expanded green cotyledons, whereas in others these had not yet emerged from the testa). By the 7th day the hypocotyls had lengthened and the cotyledons were expanded in all plants. Elongation of the hypocotyl and cotyledon expansion continued until the 9th day. In the 12-day-old seedlings the first leaf was visible and at the 16-day stage this had enlarged considerably and a second leaf had appeared. The cotyledons remained green and expanded at the end of the experimental period (16 days).

Values for fresh and dry weights are shown in Tables I and II. A normal steady increase in cotyledon fresh weight occurs between the 4th and 12th days of germination, and then there is no further rise. The maximum rate of increase is from the 6- to 7-day stage, during which time the cotyledons are expanding most rapidly. On the other hand the dry weight of the cotyledons remains approximately constant until the 6th day. It is of interest that there is a slight rise in total dry weight from 0 to 4 days. Further experiments are needed to establish the effect with certainty, though it is to be noted that similar results have been recorded for *Cucurbita* by Jegerow (1906) and Heumann (1944). A very low respiratory quotient has been found for *Citrullus* in these early germination stages (Williams and Sharma, 1954) which would allow for a small increase in dry weight if either oxygen-rich compounds were being synthesized, or CO₂ was being fixed in the dark.

Between 6 and 7 days a fall in dry weight accompanies a spurt in water content increase. After 7 days the dry weight increases steadily, photosynthesis presumably occurring in the green tissue. The fresh weight of the 'rest of the plant' increases consistently from the 4th day. Dry weight also increases generally although the rapid rise from the 6th to 7th days is followed by a slight decrease.

Light petroleum extract. Weights of light petroleum extracts are shown in Tables I and II and in Fig. 1. A slight decrease in lipid material between 0 and 4 days is accompanied by a rise in fat-free material, thus accounting for the increase in dry weight noted previously. The most noticeable feature in the cotyledon values is the sudden decrease in lipid content from the 6- to 7-day stages, which is accompanied by a rapid increase in fat-free dry weight.

The amount of extract from the 'rest of the plant' is always small, but there is a slight rise on the 6th day followed by a fall.

TABLE I
Composition of cotyledons (weight/100 seedlings)

Component	Age of seedlings (days)						
	0	4	6	7	9	12	16
Fresh weight (g.)	4.26 4.52	6.88 6.62	10.88 10.20	19.32 19.44	26.07 26.33	36.7 35.7	38 34
Dry weight (g.)	4.06 4.30	4.39 4.36	3.60 3.62	2.43 2.54	2.71 2.54	3.32 3.27	3.13 2.87
Light petroleum extract (g.)	2.11 1.97	2.04 2.00	1.59 1.54	0.362 0.315	0.165 ..	0.131 0.119	0.155 0.143
Unsaponifiable material (mg.)	25	10	30	52	25	17	26
Free fatty acids (mg.)	0.1	0.1	0.5	0.5	0.6	3.8	3.7
'Combined' fatty acids (mg.)	1353	1345	1115	207.1	51.1	36.3	12.0
Free + 'combined' fatty acids as % light petroleum extract	69.5	69.5	76.8	64.4	41.2	33.6	11.1
Fat-free dry weight (g.)	2.33 1.96	2.39 2.32	2.07 2.01	2.22 2.07	2.60 2.37	3.20 3.14	2.99 2.71
Reducing sugar (mg.)	..	2.5	7.7	33.6	63.0
Sucrose (mg.)	..	55.1	31.5	13.8	5.1
Starch (mg.)	..	22.8	36.5	81.2	66.2

TABLE II
Composition of 'rest of the plant' (weight/100 seedlings)

Component	Age of seedlings (days)					
	4	6	7	9	12	16
Fresh weight (g.)	0.11 0.13	11.58 10.27	25.10 24.32	34.19 37.16	49.9 50.3	58 55
Dry weight (g.)	0.028 0.022	0.77 0.75	1.61 1.61	1.47 1.50	1.81 2.16	3.17 3.06
Light petroleum extract (g.)	..	0.053* 0.055	0.034 0.018	0.007 0.001	0.009 0.014
Fat-free dry weight (g.)	..	0.72 0.70	1.59 1.57	1.49 1.46	2.15 1.79	3.17 3.06
Reducing sugar (mg.)	..	0.6	1.4	4.1	1.0	..
Sucrose (mg.)	..	1.4	1.6	1.0	2.8	..
Starch (mg.)	..	3.9	7.3	13.3	24.0	..

* Free fatty acid = 0.8 mg.; unsaponifiable material = 1.7 mg.; 'combined' fatty acids = 9.1 mg.

Free fatty acids. The acid values of the fat extract are shown in Table III and these are converted to weight of fatty acid (calculated as linoleic acid since this is the major component of the seed fat). When these weights are compared with the weights of free fatty acids extractable by sodium carbonate, it becomes clear that a major part of the acidity is due to substances other than long-chain free fatty acids. It is also obvious that these free fatty acids do not accumulate during the most rapid phase of fat breakdown.

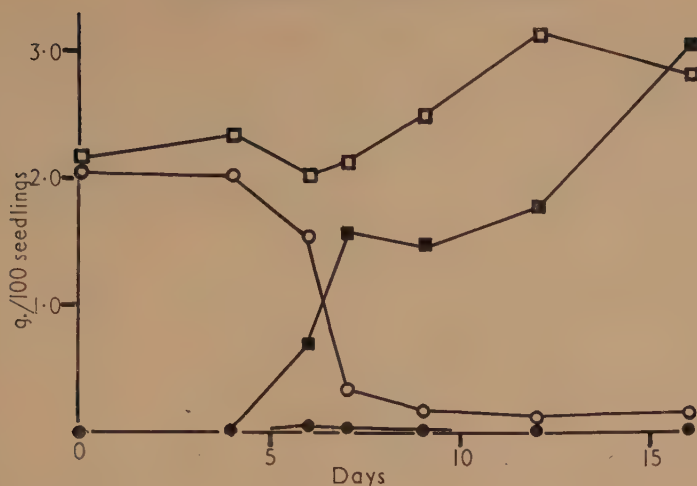


FIG. 1. Light petroleum extract and fat-free dry weight of cotyledons and 'rest of the plant'. —○— Petroleum extract (cotyledons). —□— Fat-free dry weight (cotyledons). —●— Petroleum extract ('rest of the plant'). —■— Fat-free dry weight ('rest of the plant').

TABLE III

Comparison of extractable free fatty acids with values calculated from titration of the oil

Age of seedling (days) and part analysed	Acid Value	Mg. linoleic/100 seedlings (calculated from titration of oil)	Mg./100 seedlings free fatty acids by Na ₂ CO ₃ extraction
0, Cotyledons	0.48	5.0	0.1
4, Cotyledons	0.40	4.2	0.1
6, Cotyledons	0.48	3.8	0.5
6, 'Rest of plant'	9.4	2.5	0.8
7, Cotyledons	6.7	11.1	0.5
9, Cotyledons	16.5	14.1	0.6
12, Cotyledons	32.0	20.9	3.8
16, Cotyledons	25.0	18.3	3.7

Unsataponifiable material. The weights of unsaponifiable material, both in the cotyledons and in the 'rest of the plant', are low and any changes are of doubtful significance.

'Combined fatty acids.' Weights of 'combined fatty acids' are given in Tables I and II. In the cotyledons the rapid decrease between the 6th and 7th days corresponds to a similar decrease of total lipid extract. Sufficient material was available for only a single estimation on the 'rest of the plant' and this is shown in Table II. In Table I the total fatty acids have also been expressed as a percentage of the fat extract and these figures demonstrate the increasing proportion of non-fatty acid-containing substances in the later stages of development. They also suggest that, even in the seed oil, substances other than triglycerides are present, since if the 'combined fatty acid' figure is calculated as triglyceride, all the extract is by no means accounted for.

Fat-free dry weight. Values for fat-free dry weight are given in Tables I and II and Fig. 1. A rapid rise occurs from the 6th to 7th days in the cotyledons, corresponding to a fall in weight of fat. The results of Williams and Sharma (1954), on germinating *Citrullus* of the same variety as that used in this work, indicate only small changes in total nitrogen during this phase: the major change is therefore assumed to be in carbohydrate content.

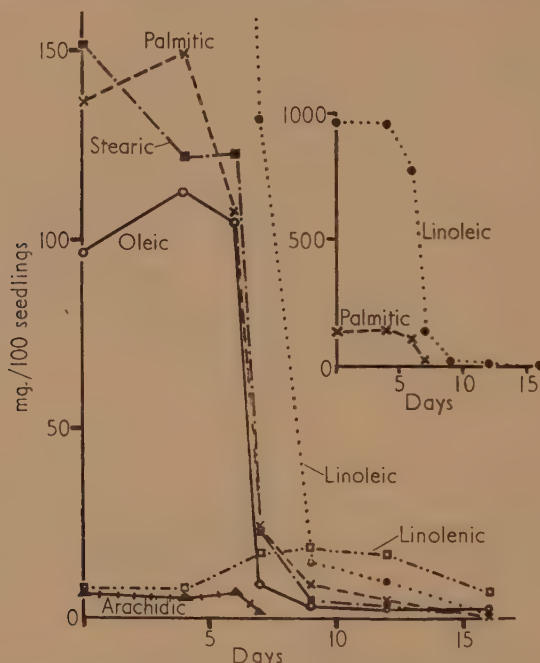


FIG. 2. Total ('combined' + free) fatty acids of the cotyledons.

Reducing sugars increase consistently in the cotyledons from the 4th to the 9th day and during the same period sucrose decreases by about the same amount. Starch shows a rise up to the 7th day and then declines. The amounts are extremely small at all times and a major fraction of the increasing fat-free dry weight must be presumed due to other compounds such as cell wall materials.

No conclusions can be drawn in the present investigation on the formation of carbohydrates from fat owing to the possibility of photosynthesis after the 4th day. This problem is under current examination.

In the 'rest of the plant' the amounts of sucrose and reducing sugars are very low although starch shows a steady increase between 6 and 12 days.

Fatty acid analysis of the seed oil. A number of fatty-acid analyses for *Citrullus vulgaris* seed oil, all using non-chromatographic methods, are recorded in the literature (Pierarts, 1917; Nolte and Loesecke, 1939; Rankov and Popov, 1941; Carriere and Coulier, 1942; Dhingra and Biswas, 1945;

Alpar and Esin, 1949; Grindley, 1950; T-Sao and Potts, 1952; and Chowdhury *et al.*, 1953). The most reliable of these use ester fractionation techniques which are inapplicable to small amounts of biological material. Survey of the earlier work indicates that varietal differences are great. Our chromatographic analysis (Table IV) of seed oil (cotyledons and embryo combined) of the variety Tom Watson shows that it most resembles another American variety, Cuban Queen (Nolte and Loesecke, 1939).

TABLE IV

Composition of 'combined' fatty acids (mg./100 seedlings, weight percentage in parentheses)

Age of seedling (days) and part analysed	Saturated acids			Unsaturated acids		
	Palmitic	Stearic	Arachidic	Oleic	Linoleic	Octadeca- trienoic (by chroma- tography)
0, Cotyledons + 'rest of plant'	136.2 (10.1)	151.0 (11.2)	7.5 (0.6)	96.9 (7.2)	961.5 (70.6)	7.2 (0.4)
4, Cotyledons	149.3 (11.1)	122.0 (9.1)	5.6 (0.4)	112.8 (8.4)	955.0 (71.0)	..
6, Cotyledons	107.2 (9.6)	122.7 (11.0)	6.9 (0.6)	104.8 (9.4)	773.5 (69.4)	..
6, 'Rest of the plant'	2.0 (21.8)	0.8 (8.6)	0.1 (1.6)	0.3 (3.1)	4.9 (53.4)	1.1 (11.6)
7, Cotyledons	24.2 (11.7)	22.8 (11.0)	1.7 (0.8)	8.7 (4.2)	131.9 (63.7)	17.7 (8.6)
9, Cotyledons	8.6 (16.8)	4.4 (8.6)	..	3.7 (7.2)	14.7 (28.8)	19.7 (38.6)
12, Cotyledons	4.6 (12.7)	2.9 (8.0)	..	2.2 (6.1)	9.7 (26.7)	16.9 (46.6)
16, Cotyledons	0.3 (2.5)	0.6 (5.0)	..	2.1 (17.5)	1.5 (12.5)	7.5 (62.5)
Leaf, 'combined' + free fatty acids.	(17.2)	(6.1)	..	(7.2)	(14.7)	(54.8)

In addition to chromatographic estimation, linoleic acid was also determined by ultra-violet light absorption after alkali isomerization (Brice *et al.*, 1952). This gave satisfactory agreement, a value of 68.9 per cent. being obtained compared with 70.6 per cent. estimated chromatographically.

Very careful chromatography of a large quantity of 'combined fatty acids' indicated the presence of a small proportion of an acid (0.36 per cent.), which was eluted prior to linoleic; from its position this was possibly an octadecatrienoic acid. Confirmation of this came from the ultra-violet light absorption spectrum of the fatty-acid mixture after alkali isomerization, which showed maxima at 268 and 278 $m\mu$ and minima at 262 and 274 $m\mu$ characteristic of a conjugated triene. The maximum at 258 $m\mu$, also characteristic of a conjugated triene, was obscured by the large quantity of linoleic acid (Comber, 1955). These results are compatible with the presence of either a conjugated (e.g. elaeostearic) or a non-conjugated (e.g. linolenic) octadecatrienoic acid

in the oil prior to isomerization. At least some conjugated¹ octadecatrienoic acid must be present originally, since the spectrum of the unisomerized oil has maxima at 258, 269, and 278 m μ and minima at 262 and 274 m μ . Background interference was considerable and partly obscured the peak at 278 m μ (Comber, 1955). Using the correction factor of method Cd 7-48 (American Oil Chemists Society, 1953) the value for conjugated acid was found to be 0.062 per cent. and of non-conjugated acid 0.084 per cent. (if no background corrections are applied the values rise to maxima of 0.54 and 0.36 per cent. respectively).

Considerable care was taken to establish these figures in order to show that the 'linolenic' acid² present in the oil from later germination stages must have been synthesized in the seedling.

Changes in individual fatty acids during germination. At the 4-day stage of germination the total amount of 'combined fatty acid' in the cotyledons is approximately the same as that in the ungerminated seeds (Table I, Fig. 2). The composition differs slightly, the mixture containing more palmitic and oleic and less stearic acids; linoleic, the major component acid, remains constant. It was not possible to determine the composition of the free fatty acids from these early stages, owing to the very small amounts obtained.

In the cotyledons of the 6-day seedlings, all the major 'combined' fatty acids have decreased proportionally (Table IV). The corresponding fraction from the 'rest of the plant' shows a marked difference in composition. 'Linolenic' acid forms a measurable percentage of the mixture; oleic, linoleic, and stearic acids form a smaller proportion than in the cotyledons, and palmitic and arachidic acids a larger. The total quantities are low, indicating little accumulation in any part of the seedling other than the cotyledons.

The rapid decrease in weight of the 'combined fatty acids' in the 7-day cotyledons is accompanied by surprisingly small changes in its fatty acid composition, although oleic acid has disappeared more rapidly than the other major components. There is a definite increase in the amount of 'linolenic' acid present. Despite the rapid loss of oil between the 6th and 7th day stages, no accumulation of free fatty acids occurs.

By the 9th day the amount of 'combined fatty acid' in the cotyledons is very low (approximately 4 per cent. of that in the dry seed). 'Linolenic' acid is now a major component and has increased in weight (per cotyledon) from the preceding stage (see Table V).

At 12 days the composition of cotyledon 'combined fatty acids' is similar to that at 9 days although there is a weight decrease. Free fatty acids were

¹ It was suspected that the ester linkage might cause absorption in the same region, and to test this purified trimyristin was examined. The results showed that very little absorption was due to this factor (approximately one-tenth that of the water-melon oil).

² Chemically, linolenic acid is octadeca-9:12:15-trienoic acid. However, since other non-conjugated octadecatrienoic acids might be expected to behave spectroscopically (after isomerization), and chromatographically in a similar way to linolenic acid, we have referred to the triene in inverted commas. There is no chemical evidence to give a clear decision on the location of the ethylenic linkages in the chain.

analysed at this stage with the results shown in Table VI. The mixture is slightly different from that of the 'combined fatty acids' of the same age, there being relatively more oleic and less linoleic acid in the free fatty acids.

TABLE V

Cotyledon octadecatrienoic acids (mg./100 seedlings). (Owing to the extremely small quantities involved and the difficulties in applying background corrections, the error in these determinations is high and the results are of qualitative significance only (see text))

Age of seedling (days)	Total C ₁₈ triene (by chromatography)	Conjugated C ₁₈ triene (U.V.)	Non-conjugated C ₁₈ triene = 'linolenic' (U.V.)
0	4.8 } 7.2 9.6 }	1.2	1.7
4	..	1.4	0.2
7	17.7	6.2*	..
9	19.7	0.05*	..

* Maximum values without correction for background absorption.

TABLE VI

Composition of cotyledon free fatty acids (mg./100 seedlings, weight percentages in parentheses)

Age of seedling (days)	Saturated acids		Unsaturated acids		
	Palmitic	Stearic	Oleic	Linoleic	Octadecatrienoic
12	0.3 (8)	0.5 (13)	0.9 (24)	0.4 (11)	1.7 (45)
16	0.9 (24)	0.3 (8)	0.3 (8)	0.9 (24)	1.3 (35)

The 16-day old cotyledons had a very low content of 'combined fatty acids' and these contained relatively more 'linolenic' and less linoleic acid than the previous stage. Free fatty acids were also analysed and contained proportionally more linoleic and palmitic and less oleic acid than the 'combined fatty acids'.

Changes in percentage fatty acid composition of the oil from different stages can be seen in Fig. 3.

DISCUSSION

The cotyledons of *Citrullus vulgaris* seedlings behave similarly to those of *Cucurbita* and *Helianthus* in exhibiting a most surprising decrease in fat content as they emerge from the testa and expand. In this investigation there was a fall from 1.57 gm. of extract per 100 seeds to 0.34 gm. in a single day (Table I, Fig. 1). This remarkably rapid change is accompanied by a corresponding increase in fat-free dry weight, but as these experiments were conducted in the light no definite conclusions on interconversions can be drawn.

The general level of activity of the cotyledons at this time is great, cell extension and chlorophyll production are at a maximum and there is a high respiration rate (Williams and Sharma, 1954).

According to older theories fat breakdown is initiated by lipase which liberates free fatty acids, these then being degraded by desmolase enzymes. On this basis free fatty acids will accumulate if the desmolase is less active

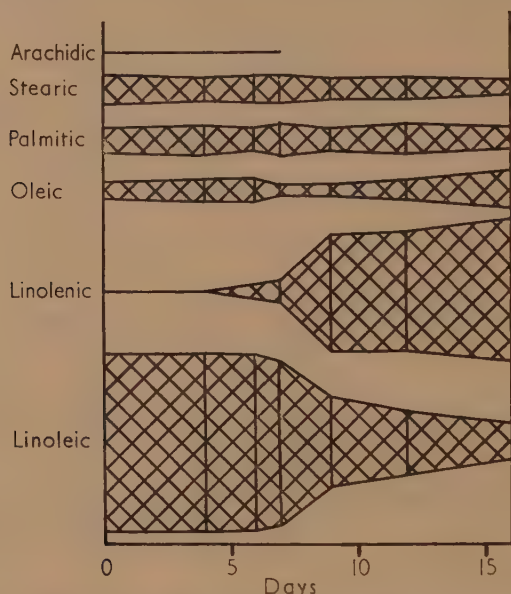


FIG. 3. Weight percentage composition of total ('combined' + free) fatty acids of the cotyledons.

than the lipase. Heumann (1944), using *Cucurbita pepo*, commented on the small rise in acidity of the oil during its phase of most rapid disintegration, and suggested that this plant must contain very active desmolase enzymes. A similar result was obtained for sunflower by Miller (1910, 1912), who reported little rise in acid value until two-thirds of the fat had disappeared. In later stages, however, he found particularly high values and used these to support the view that fatty acids are the first liberated products of fat breakdown.

In the present work on *Citrullus* the rise in free acidity of the oil during the early germination stages is also small (Table III), but high acid values are recorded later. We have, however, shown that only a fraction of this acidity is due to extractable free fatty acids. Examination of an extract from 12-day cotyledons showed that the remaining acidity is not to be accounted for by water-soluble substances. Electrometric titration in 95 per cent. ethanol indicated the presence in the oil of a very weakly acidic substance, and a plausible explanation is that the acid value unaccounted for by isolatable free

fatty acids is due to titration of a phosphatide, such as cephalin, with alkali-binding properties (Jukes, 1934; Fischgold and Chain, 1934, 1935). Phosphatides are known to increase in amount during seedling development in the light (Hée and Bayle, 1932; Houget, 1942; Smith, 1947) and the oil from the later germination stages may well contain greater proportions of these components. The many conflicting reports on acid values recorded in the literature (*vide* Muntz, 1871; Du Sablon, 1895; Green, 1890; Green and Jackson, 1905; Von Furth, 1904; Jegerow, 1904; Deleano, 1909, 1910; Matthes, 1927; Johnston and Sell, 1944) may result from a similar cause.

We have obtained no evidence for the accumulation of free shorter-chain fatty acids during rapid fat breakdown, although the oil has been examined for volatile fatty acids at this stage. The presence of short-chain acids was inferred by Miller (1910, 1912) from changes in saponification values, but in the light of our findings that the petrol extracts contain considerable amounts of substances other than glycerides, it would seem that this work requires re-examination.

We conclude that *Citrullus* has a particularly active fat metabolism and that any acidic intermediates are not accumulated.

No very definite evidence of fatty acid interconversions has been obtained. In the first 4 days of germination, oleic and palmitic acids increased slightly, whereas linoleic and stearic acids decreased (a similar rise in oleic acid at this stage was noted by Heumann (1944) in *Cucurbita*). During the very rapid disintegration of the storage oil all the acids except oleic decrease proportionally, there being some evidence that the latter acid disappears relatively faster (Table VII). It is perhaps relevant that oleic acid has been shown by Simmons and Quackenbush (1954 *a, b*) to be the first-formed acid in soya beans. Heumann (1944) reports for *Cucurbita* that the oil does not change appreciably in constitution until a large proportion has disappeared and similar results have been obtained in tung by Johnston and Sell (1944) and in castor oil by Houget (1942, 1943).

TABLE VII

Rates of disappearance of fatty acids from the cotyledons

% change of individual acids between different stages (based on the weight of each acid present at 0 days)

Fatty Acid	0-4 days	4-6	6-7	7-9
Linoleic	-1	-19	-67	-12
Oleic	+16	-8(-7*)	-99(-92*)	-5
Palmitic	+10	-30	-61	-12
Stearic	-19	+1	-66	-12
% fat remaining . . .	99.0	76.5	16.9	8.1

* Calculated on the amount in the previous stage.

Following and during the disappearance of the major portion of the storage fat there is a gradual synthesis of 'linolenic' acid as the cotyledons expand and turn green. This leads to a marked change in composition of the oil

(Fig. 3) which shows increasing unsaturation. In contrast, a decrease in unsaturation during the later stages of germination has been recorded, from iodine values, by Jegerow (1906) and Weber (1936) for *Cucurbita*, by Miller (1910, 1912) and Tilenius (1938) for *Helianthus*, and by Holman (1948) for *Soya*, although the last author noted no significant changes in linoleic and linolenic acids as determined spectrophotometrically. If iodine values are calculated for *Citrullus* a decrease in unsaturation is deduced for this plant too; the discrepancy results from the increasing proportion of non-fatty acid-containing substances in the light petroleum extract (Comber, 1955).

The results suggest that two processes are occurring in the cotyledons during germination in *Citrullus*. First, a rapid breakdown of the storage oil, and secondly the synthesis in small quantities of a different oil associated with the photosynthetic function. It is noteworthy that the fat from fully expanded (16-day) cotyledons closely resembles that from mature *Citrullus* leaves (Table IV), both in fatty acid composition (within the limits of experimental error for the particular determinations) and in total quantity per gm. dry weight of tissue. It is also similar to other leaf fats (*vide* Hilditch, 1949, p. 139).

The present investigation has thus traced for the first time transition from the fatty-acid composition typical of a storage organ to that typical of a fully expanded and photosynthesizing leaf.

ACKNOWLEDGEMENTS

We are grateful to Professor W. T. Williams for his constant interest and encouragement during the progress of this work and thank the Ferry Morse Seed Company of California for their generous gift of *Citrullus vulgaris* seed.

One of us (R. C.) acknowledges the receipt of a maintenance grant from the University of Southampton.

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Fat Metabolism in the West African Oil Palm (*Elaeis Guineensis*)

PART I. FATTY ACID FORMATION IN THE MATURING KERNEL

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SUMMARY

Using a column chromatographic technique for the estimation and identification of fatty acids, a study has been made of fat formation in developing Oil Palm kernels from 10 weeks after pollination to full maturity (20 weeks), during which time the fat content may increase a hundredfold. Nuts from three different trees have been analysed and differences between these in rates of maturation (as indicated by appearance of the endosperm) can be directly correlated with changes in character of the oil present.

Amounts of reducing sugars, sucrose, and starch in the developing endosperm have also been followed, but these carbohydrates are present throughout in low concentration, and it is assumed that translocation from the rest of the plant to the developing kernel must account for the major part of storage material.

The mature kernel contains in its fat an unusual mixture of eight different saturated fatty acids. The major such component is lauric acid (46.1 to 49.5 per cent.) and there are present two common unsaturated acids, oleic (15.7 to 16.5 per cent.) and linoleic acid (0.7 to 3.1 per cent.). At the earliest stage examined (10 weeks after pollination) all these acids are present but in altered proportions, unsaturated acids forming a larger fraction (36.5 to 81.2 per cent., according to the tree investigated), and lauric acid (1.4 to 8.5 per cent.) a smaller.

The results suggest that young kernels contain a small quantity of a largely unsaturated 'protoplasmic' fat, and that at a certain stage in development some physiological change in the tissue results in the formation, in large quantities, of a new, highly saturated storage fat. No fatty acid interconversions could be demonstrated although there is some suggestion that oleic acid behaves anomalously.

There is evidence that free fatty acids are not accumulated prior to esterification.

INTRODUCTION

THE present paper is intended as the first in a series which will consider various aspects of fat metabolism in the West African Oil Palm (*Elaeis guineensis* var. *dura*). Fat formation and fat breakdown have seldom been investigated in the same plant and knowledge of oil metabolism in monocotyledons is extremely scanty. The Oil Palm is of particular interest in this respect as it forms two completely different fats, both of which are of considerable economic importance. Palm oil is formed in the pericarp and has a composition similar to many other fruit-coat oils, being relatively unsaturated, whereas the endosperm oil (palm kernel oil) contains a most unusual and complex mixture of saturated fatty acids. By using this plant it has therefore been possible to study

concomitant formation, in adjacent tissues, of two different oils, one predominantly unsaturated, the other predominantly saturated. An examination is also being made of fat breakdown in the endosperm following and during germination, and the formation, at the same time, in the young seedlings of root and shoot oils.

A large number of papers record the course of fat formation in a variety of different seeds (*inter al.* du Sablon, 1896; McClenahan, 1913; Garner, Allard and Foubert, 1914; Dillmann, 1928; Caskey and Gallup, 1931; Thor and Smith, 1935; Stark 1924; Sell, Johnston, and Lagasse, 1946; and Pickett, 1950), but in the majority of these investigations changes in oil composition have been inferred from indirect data such as acid, saponification, and iodine values. These methods were developed primarily for use on oils from mature seeds, and can give most misleading results when applied to extracts containing only minor proportions of fatty acid-containing substances, such as are encountered in immature seeds.

Individual fatty acids have been infrequently estimated; Painter (1944) recorded changes during flaxseed maturation in total saturated acids, oleic, linoleic, and linolenic acids, and Grindley (1950) recorded changes in saturated acids, oleic and linoleic acids during ripening of cottonseed. Both these authors used methods dependent on the determination of suitable constants (iodine and thiocyanogen values, &c.) on the extracted fatty acids. Although amounts of individual unsaturated fatty acids can be inferred, these methods do not allow the component saturated acids to be estimated or identified.

The most recent and most complete investigation is that of Simmons and Quackenbush (1954*a* and *b*) on developing soya beans, in which fatty acids (total saturated, oleic, linoleic, and linolenic acids) were determined by methods involving adsorption chromatography and ultraviolet light absorption. Use was also made by these authors of radioactive tracers and their results will be referred to later.

In the present study a reversed-phase column chromatographic method, suitable for estimation and identification of milligram quantities of fatty acids, has been used throughout.

METHODS

Plant material. Developing fruits of the Oil Palm were supplied by the West African Institute for Oil Palm Research (WAIFOR), Nigeria, through the courtesy of the Director, Mr. F. W. Toovey. Preliminary investigations had indicated that there were differences in fat composition of kernels from different trees, even those of the same variety. Rates of fat formation also varied according to the season at which maturation occurred. Oil Palms are in fruit throughout the year in Nigeria, for example, ripening in March 1955, as measured by fat content and composition, was found to be about 2 weeks in advance of that in October 1953. There are also known to be considerable differences in maturation rates between fruits of different bunches, and also between those in different positions on the same bunch (Blommendaal, 1925).

In this investigation fruits were picked from three trees of similar variety (dura), which formed part of a WAIFOR genetical experiment. The bunches used had all been artificially pollinated at the same time and samples were removed from the centres of bunches only in order to obviate the variation due to positioning of the fruit. Preliminary experiments had indicated that the time range for active fat formation, both in the mesocarp and in the endosperm, was from 10 weeks after pollination to maturity (20 weeks), and material was accordingly collected on the same dates (corresponding to 10, 11, 12, 13, 14, 15, 16, 19, and 20 weeks after pollination), from all three trees. Each sample comprised 20 nuts which were freed from their pericarps at WAIFOR and sent to England preserved in acetone (this solvent being used in preference to alcohol owing to the possibility of esterification of free fatty acids in the latter). The corresponding pericarp material was preserved in a similar way, and the results will be reported later.

Dry weight, and light petroleum extract. The preserved nuts were sawn in half and their kernels, including the testas, carefully removed. The acetone solution was evaporated and the residue added to the kernels. Drying was effected at 80° C. in a nitrogen atmosphere to constant dry weight. After grinding in a mortar, the dried material was continuously extracted for 6 hours in a Soxhlet apparatus with light petroleum (b.pt. 40–60° C.). The kernels, particularly those of the later stages, were extremely difficult to grind and, in some cases, after partial extraction, the material was re-ground and re-extracted to give a total extraction time of 6 hours.

Free fatty acids. After removal of solvent at low temperature, the acidity of the light petroleum extract was determined by titration with 0.05N NaOH in ethanolic solution to a phenolphthalein end point (pH 9.4). The ethanol was removed and the material partitioned between light petroleum (b.pt. 40–60° C.), and dilute sodium carbonate solution. The combined aqueous solutions were acidified with dilute HCl and the liberated free fatty acids extracted into light petroleum and washed with water. After drying (Na_2SO_4), and evaporation of the solvent, the residue was titrated with 0.01N ethanolic NaOH.

This procedure determines only longer-chain fatty acids (chain length $> C_6$), as the shorter-chain volatile acids, being water-soluble, will be partly lost during the extraction. Volatile free fatty acids were determined by Method Cd 5–40 of the American Oil Chemists' Society, 1953.

Non-saponifiable material and combined fatty acids. The saponification method has already been described (Crombie and Comber, 1956). Neutral non-saponifiable material was extracted in ethyl ether, dried (Na_2SO_4), and weighed, and the aqueous soaps acidified, extracted with light petroleum, dried (Na_2SO_4), the solvent evaporated, and weighed as 'combined fatty acids'. Individual fatty acids were determined by a reversed-phase column chromatographic procedure (Crombie, Comber, and Boatman, 1954, 1955). Linoleic and linolenic acids were estimated in a single sample by ultraviolet light absorption following alkali isomerization (Brice *et al.*, 1952), using a Hilger photoelectric Uvispek (Crombie and Comber, 1956).

Iodine values were determined on the 'combined fatty acids' by Wijs's method (30 minutes) using approximately 30 mg. of material in 0.8 ml. CCl_4 with 1 ml. 0.2N Wijs's solution, and titrating the iodine liberated by KI with 0.01N sodium thiosulphate.

Carbohydrates. Starch, reducing sugars, and sucrose were estimated by methods described previously (Crombie and Comber, 1956).

RESULTS

Appearance of the kernels. In the earliest stages of development the endocarp is filled with an aqueous solution which contains practically no fat; as ripening progresses the contents gradually solidify, becoming at first gelatinous and later very hard and oily. The three trees examined show some differences in their rates of kernel development, tree 6—140 being slowest and 6—168 fastest (Table I). A further source of variation arises from the differential production of 2- and 3-seeded nuts; the latter are larger than the normal type but may contain smaller individual kernels. The results have been recorded on a per nut basis and no account taken of this factor.

Differences in appearance between individuals of a single sample were remarkably slight, indicating that the careful selection of fruit from the same position on bunches of the same age had successfully removed these sources of variation. As all material was collected at the same time, any changes noted should therefore be characteristic for the particular tree studied.

Dry weight. In all three trees there is a considerable overall rise in dry weight during the period studied (Table II), although the actual dry weights per nut differ considerably in the three examples. Tree 6—140 has a consistently lower dry weight at all stages examined than either 6—168 or 6—173, although the high dry weight of the latter is partially explained by the large numbers of 2- and 3-seeded nuts which it produces. This latter fact is almost certainly responsible for the apparent loss in dry weight between 13 and 14 weeks in 6—173, and the fluctuation in the curve for tree 6—168 between the 15 and 16 week stages.

Light petroleum extract. Ten weeks after pollination the weight of light petroleum extract in the kernels is extremely low, varying from approximately 11 mg. per nut in trees 6—168 and 6—173 to only 2.5 mg. per nut in tree 6—140. This fraction increases slowly from 10 to 12 or 14 weeks and then rises rapidly as the bulk of storage fat is laid down. The three trees show differences in the time at which the fat begins to amass, this being at 12 weeks after pollination in 6—173, 13 weeks in 6—168, and between 13 and 15 weeks in tree 6—140 (Table II). At maturity (20 weeks) about 40 per cent. of the dry weight consists of fat in all three examples, although there are differences in the weights of fat per nut produced by the three trees, tree 6—140 giving the lowest yield of extract per nut and 6—173 the highest. These results are at least partly due to the large numbers of 2-seeded nuts formed in tree 6—173.

Carbohydrates. These were determined in only two of the three sets of nuts examined, and in both cases the amounts of the three carbohydrates estimated

TABLE I

Appearance and Numbers of Kernels at Different Times after Pollination

(20 nuts per sample throughout)

Weeks from pollination	Tree 6—168		Tree 6—173		Tree 6—140	
	Appearance of kernel	No. of 2-seeded nuts	Appearance of kernel	No. of 2-seeded nuts	Appearance of kernel	No. of 2-seeded nuts
8	Liquid	1	—	—	—	—
10	Semi- gelatinous	0	Semi- gelatinous	4	Liquid	0
11	Gelatinous	3	Semi- gelatinous	8	—	—
12	Gelatinous	3	Gelatinous	6	—	—
13	Harder, but still gelatinous	0	Harder, but still gelatinous	10	Semi- gelatinous	1
14	Harder, but still gelatinous	2	Harder, but still gelatinous	2	—	—
15	Hard	2	Hard	7*	Harder, but still gelatinous	2
16	Hard	0	Hard	4	—	—
19	Hard	2	Hard	4	—	—
20	Hard	3	Hard	5	Hard	2

* In addition, 2 3-seeded nuts.

(starch, sucrose, and reducing sugars) were low at every stage, forming only a small proportion of the fat-free dry weight. Both starch and sucrose accumulated to a slight extent during the later stages of maturation, but the behaviour of the reducing sugars was different in the two examples. In tree 6—173 these remained at a low, fairly constant level between 12 and 20 weeks, being almost absent in the earliest stages, whereas in tree 6—168 reducing sugars were at a higher level in the 10 to 12 week stages, falling later to a very low value (Table II).

Free fatty acids. Titration figures for light petroleum extracts of kernels at the different stages are given in Table III as acid values (mg. KOH needed to neutralize 1 g. of oil), and although the results show considerable variation, it is clear that acid values are markedly higher in the 10-week stage and fall with advancing maturity. These figures have also been used to calculate weights of free fatty acids using the molecular weight of lauric acid, since this is the major component of the mature seed oil. It can be seen from these figures that free fatty acids constitute only a very minor fraction of the oil at the time when it is being most actively laid down.

In the same table are shown for comparison weights of sodium carbonate-extracted free fatty acids, also calculated as lauric acid. It is at once apparent that, particularly in the early stages, a major part of the acidity of the light petroleum extract is due to substances other than long-chain ($> C_6$) fatty

TABLE II
Changes in Components during Seed Maturation

(weights in mg./nut)								
Weeks after pollination	Dry wt.	Light petroleum extract	Non-sapon.	'Combined* fatty acids' (total wt.)	Starch	Sucrose	Reducing sugars	Fat-free dry wt.
Tree 6—168								
10	73.4	10.9	0.5	7.7 (70 %)	0.3	0.0	12.1	62.5
11	133.6	18.5	1.0	15.6 (84 %)	3.5	1.5	18.9	115.1
12	159.5	32.0	0.5	22.7 (71 %)	1.7	0.1	9.2	127.5
13	198.5	61.5	1.2	49.4 (81 %)	0.8	2.4	2.4	137.0
14	383.0	106.8	0.9	86.8 (81 %)	0.9	1.7	3.4	276.2
15	591.2	283.8	0.4	244.0 (86 %)	0.8	1.7	0.7	307.4
16	613.0	—	—	—	2.9	0.2	0.3	—
19	1076.0	408.0	0.3	374.0 (93 %)	5.2	9.3	0.9	668.0
20	1208.0	463.0	2.7	417.0 (90 %)	26.7	9.3	2.1	745.0
Tree 6—140								
10	—	2.5	0.1	1.7 (66 %)	—	—	—	—
13	137.4	32.7	0.1	22.9 (70 %)	—	—	—	104.7
15	434.3	175.2	0.1	147.8 (84 %)	—	—	—	259.1
20	949.1	341.5	0.1	319.5 (94 %)	—	—	—	607.6
Tree 6—173								
10	92.8	12.6	0.8	10.0 (79 %)	0.4	0.1	0.0	80.2
11	118.5	27.4	0.6	16.9 (62 %)	0.9	0.9	2.7	91.1
12	302.3	53.4	0.3	—	1.6	0.5	6.7	248.9
13	561.0	175.5	6.3	138.1 (79 %)	2.0	0.0	4.8	385.5
14	537.2	198.4	0.4	169.5 (86 %)	1.5	0.3	7.3	338.8
15	757.7	294.1	—	—	1.6	0.5	5.8	463.6
16	930.8	441.0	1.1	423.4 (96 %)	2.1	0.4	5.4	489.8
19	1112.2	512.5	—	—	3.8	1.6	6.0	600.2
20	1463.2	569.9	2.3	529.0 (93 %)	8.1	21.4	6.0	893.3

* Figures in brackets represent 'combined fatty acids' as per cent. of the light petroleum extract.

acids. There is therefore no evidence to support the theory that such long-chain free fatty acids accumulate prior to esterification. As has been made clear earlier (see Methods), volatile free fatty acids are lost during the sodium-carbonate extraction procedure, and it was therefore possible that such acids were responsible for a major part of the acidity. In order to test this an examination was made of oil from a later sample (12 weeks after pollination, 1955) of kernels, which gave the following titration results per 1/25 of light petroleum extract: original extract \equiv 4.80 ml. 0.005N NaOH, sodium carbonate extract \equiv 0.25 ml., water solubles \equiv 0.00 ml., steam volatile fatty acids \equiv 0.08 ml. From these results it seems unlikely that shorter-chain free fatty acids are responsible for more than a minor fraction of the acidity, although further data are required to substantiate this finding. It is therefore assumed that acidic substances other than free fatty acids are present in the light petroleum extract. A similar result has been obtained in a study of germinating *Citrullus* (Crombie and Comber, 1956).

Non-saponifiable material. The weights of non-saponifiable material are shown in Table II. They are at all stages very low.

TABLE III

Free Fatty Acids (amounts per 20 nuts)

Weeks from pollination	Wt. of light petroleum extract (mg.)	Acid value (mg. KOH/g. extract)	Mg. lauric acid, calculated from A.V.	Mg. lauric acid (Na ₂ CO ₃ *)	Higher (> C ₈) fatty acids as % petroleum extract
10 6-173	252	29.8	26.8	3.4	1.4
6-140	50	91.9	17.7	—	—
11 6-173	548	17.8	34.7	5.4	1.0
12 6-168	640	9.5	21.6	0.4	0.1
6-173	1,068	11.7	44.6	10.5	1.0
13 6-168	1,230	—	—	2.4	0.2
6-173	3,510	7.5	93.5	6.0	0.2
6-140	654	7.2	16.7	13.5	2.1
14 6-173	3,968	3.3	23.1	13.9	0.4
15 6-168	5,670	—	—	19.3	0.3
6-173	5,882	3.0	30.7	—	—
6-140	3,504	—	—	19.5	0.6
16 6-173	8,820	3.0	46.7	22.5	0.3
19 6-168	8,160	0.5	6.6	0.03	0.0
6-173	10,250	4.6	83.8	—	—
20 6-168	9,260	—	—	21.0	0.2
6-173	11,398	1.2	24.0	0.0	0.0
6-140	6,830	3.3	40.0	34.9	0.6

* Free acids extracted by sodium carbonate, titrated and calculated as lauric acid.

'Combined fatty acids' (total weight). The weights of total 'combined fatty acids' (Table II) parallel closely those for the light petroleum extracts, showing a fairly steady rise between 10 and 20 weeks. As in the case of the oil extract, highest values are recorded for tree 6-173, and lowest for tree 6-140. An interesting feature is that with increasing maturation there is a parallel increase in the percentage of 'combined fatty acid' in the light petroleum extract, the value rising from about 70 per cent. in the 10-week stage to over 90 per cent. in mature kernels. Such results would be obtained if fatty acids were combined largely as phosphatides in the early stages and as glycerides later, but other explanations are possible. The values at 20 weeks correspond very closely to those calculated for triglycerides (e.g. trilaurin contains 94 per cent. fatty acids on a weight basis).

Composition of the 'combined fatty acids'. (i) *In the mature kernel.* The mature kernel oils of the three trees examined have closely similar fatty acid compositions, and contain every even-numbered straight chain saturated fatty acid from C₈ to C₁₈ inclusive. In addition, traces of the C₆ and C₂₀ acids also occur, these last being included (Table IV) with the C₈ and C₁₈ acids respectively.

Saturated acids total over 80 per cent. of the combined fatty acids, the major component being lauric acid (46.1 to 49.5 per cent. in the three examples), accompanied by smaller fractions of myristic (18.4 to 19.8 per cent.) and palmitic (6.8 to 13.3 per cent.) acids. Tree 6-168 has a rather high content of this last acid compared with the other two examples.

In addition to this unusual mixture of saturated acids two common unsaturated acids, oleic and linoleic, are also present, the latter, however, to only a minor extent (0.7 to 3.1 per cent.). A closely similar chromatographic analysis of a mixed sample of WAIFOR palm kernels (var. *dura*) has already been reported (Crombie and Boatman, 1956), and it is noted that this shows excellent agreement with values obtained for a similar oil by methyl ester fractionation (Dale and Meara, 1955).

Changes in fatty acid composition during maturation. All the major fatty acid components of the mature kernel oil are present even in the youngest stage studied (10 weeks after pollination), when the fat content may be remarkably low (e.g. only 2.5 mg. per nut in tree 6—140). The proportions of these acids are, however, very different, lauric acid forming only a minor fraction of the total in the 10 week stage, compared with nearly 50 per cent. in the mature nut. In spite of considerable variation between trees in the young stages, a general similarity in fatty acid pattern can be discerned (Table IV and Fig. 1), the chief feature being the preponderance of unsaturated acids, giving the oil a totally different character from that in mature kernels. Linoleic acid forms about 15 per cent. of the total acids in all three examples at 10 weeks and oleic acid varies from about 20 per cent. in tree 6—140 to nearly 70 per cent. in 6—168. It was possible that traces of linolenic acid might occur and to test this the mixture from tree 6—140 was examined for ultraviolet light absorption following alkali isomerization. No characteristic triene absorption maxima were obtained at 258, 268, and 278 m μ , and it is therefore established that linolenic acid is absent.

Palmitic acid is the major saturated acid present in the youngest stages, forming from 14 per cent. of the total combined fatty acids in tree 6—168 to 42 per cent. in tree 6—140 with tree 6—173 having an intermediate content. Stearic acid is present to a somewhat greater extent than in the mature kernel oil. Both myristic and lauric acids occur but, as has been noted above, in only minor concentrations, the same being true of lower saturated acids, which show no marked percentage changes during maturation.

Part of the variation between trees in the 10-week stage may result from the three examples being in different phases of development. This idea receives some support from the fact that, whereas tree 6—168 has its maximum oleic acid content at 10 weeks, this is not reached in tree 6—173 until the 11-week stage, and in the slow-developing 6—140 not until approximately 13 weeks after pollination. Examination of the data in Table I will show that these changes can be directly correlated with the appearance of the kernels.

As ripening progresses, considerable alterations in fatty-acid composition occur, the first of these being an increase in proportion of oleic acid, which, as already mentioned, takes place at an early stage corresponding to a change in the kernels from a liquid to semi-gelatinous state. Following this there is a large increase in the proportions of both lauric and myristic acids and a corresponding decrease in oleic, linoleic, and stearic acids. Palmitic acid decreases in two examples (6—140 and 6—173) but remains approximately constant in tree

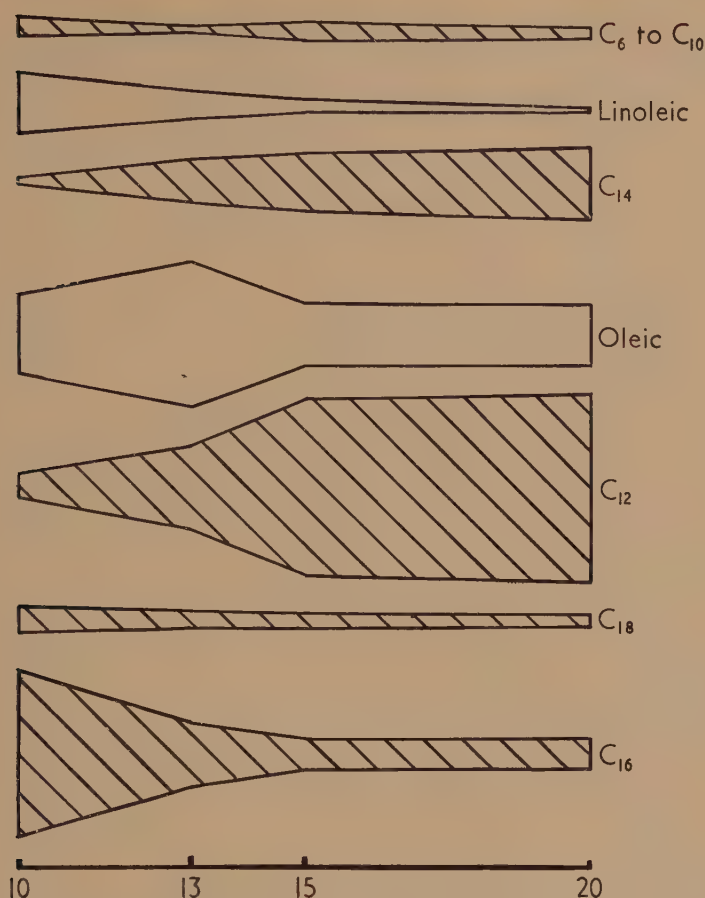


FIG. 1. Change in weight percentage composition of individual fatty acids during kernel maturation in tree 6—140. Weeks after pollination shown as abscissae.

6—168. These major changes in fatty acid composition are completed by 15 weeks after pollination in every case, and although considerable quantities of fat are formed after this time, little change in fatty-acid composition is found. It is noticeable that the alteration in type of fat produced occurs at different rates in the three examples, being completed by about 14 weeks in 6—168, 15 weeks in 6—140, and 13 weeks after pollination in 6—173.

Iodine values of the combined fatty acids have been determined in two sets of samples (6—168 and 6—173) and are shown in Table IV where they are compared with similar values calculated from the chromatographic results. Good agreement between these figures is found, there being a general decrease in unsaturation with increasing maturation.

TABLE IV
Composition of the Combined Fatty Acids

Wts. in mg./nut. Figures in brackets represent individual fatty acids as % total fatty acid

Weeks from pollination	Saturated					Unsaturated			I.V. of combined fatty acids (Wjys)	I.V. calculated from chrom. results		
	C ₈ +C ₉	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈	Oleic	Linoleic				
Tree 6—168												
10	.	.	0.03 (0.4)	0.04 (0.5)	0.11 (1.4)	0.09 (1.2)	1.02 (13.3)	0.15 (2.0)	5.16 (67.1)	1.08 (14.1)	85.4*	85.9
12	.	.	0.11 (0.5)	0.21 (1.2)	3.92 (21.4)	1.58 (8.6)	2.04 (11.1)	0.79 (4.3)	8.55 (46.5)	1.06 (6.4)	53.9	53.5
13	.	.	3.1 (6.2)	0.8 (1.7)	12.7 (25.7)	5.0 (10.1)	5.3 (10.7)	1.8 (3.6)	17.6 (35.6)	3.2 (6.4)	46.8	43.6
14	.	.	3.5 (4.0)	1.8 (2.1)	38.4 (44.3)	13.6 (15.7)	6.5 (7.5)	1.9 (2.2)	18.4 (21.2)	2.6 (3.0)	23.0	24.5
15	.	.	9.3 (3.8)	9.5 (3.9)	116.0 (47.6)	41.7 (17.1)	23.4 (9.6)	5.4 (2.2)	34.9 (14.3)	3.7 (1.5)	—	15.6
19	.	.	6.4 (1.7)	5.2 (1.4)	189.6 (50.7)	67.0 (17.9)	34.0 (9.1)	9.0 (2.4)	52.7 (14.1)	10.1 (2.7)	—	17.6
20	.	.	8.3 (2.0)	11.3 (2.7)	192.0 (46.1)	76.8 (18.4)	55.5 (13.3)	7.1 (1.7)	66.7 (16.0)	12.5 (3.1)	19.7	19.8
Tree 6—173												
10	.	.	—	—	0.85 (8.5)	0.43 (4.3)	2.82 (28.2)	0.78 (7.8)	3.52 (35.2)	1.44 (14.4)	—	41.2
11	.	.	—	—	0.16† (1.6)	—	—	—	—	—	—	—
12	.	.	0.6 (2.3)	0.5 (2.0)	8.8 (34.4)	3.1 (13.4)	2.8 (13.7)	1.1 (5.0)	8.8 (28.1)	0.3 (1.1)	20.7	28.3
13	.	.	6.8 (4.9)	4.6 (3.3)	66.7 (48.3)	18.2 (13.2)	10.2 (7.9)	5.0 (3.6)	18.4 (13.3)	3.3 (2.4)	15.1	16.3
14	.	.	4.6 (3.3)	0.4 (0.3)	68.2 (48.4)	20.0 (14.2)	10.7 (7.6)	4.1 (2.9)	26.4 (18.7)	6.5 (4.6)	23.6	25.1
16	.	.	14.0 (3.3)	15.2 (3.6)	204.0 (48.3)	87.5 (20.7)	40.6 (9.6)	6.3 (1.5)	52.5 (12.4)	4.3 (1.0)	16.9	13.0
20	.	.	14.8 (2.8)	13.2 (2.5)	256.0 (48.3)	105.0 (19.8)	36.0 (6.8)	12.7 (2.4)	87.3 (16.5)	3.7 (0.7)	16.0	14.3
Tree 6—140												
10	.	.	—	—	0.1 (6.5)	0.03 (1.8)	0.72 (43.7)	0.1 (6.5)	0.34 (20.7)	0.26 (15.8)	—	47.2
13	.	.	0.5 (0.2)	0.5 (0.2)	5.0 (22.2)	2.6 (11.2)	3.8 (16.7)	1.0 (4.6)	8.5 (37.2)	1.8 (7.7)†	—	47.3
15	.	.	2.8 (2.0)	5.2 (3.7)	65.7 (46.7)	21.0 (15.0)	11.7 (8.3)	5.9 (4.2)	23.3 (16.6)	4.9 (3.5)	—	21.2
20	.	.	0.32 (0.1)	8.6 (2.7)	153.0 (49.5)	59.5 (18.6)	30.4 (9.5)	9.9 (3.1)	50.0 (15.7)	2.6 (0.8)	—	15.5

* I.V. at 11 weeks = 63.6, † Calculated as C₁₈.
‡ By U.V. 8.3 per cent.

* I.V. at 11 weeks = 63.6.

† Calculated as C₁₈.

‡ By U.V. 8.3 per cent.

DISCUSSION

The chromatographic method used in this investigation, since it enables a full analysis on milligram quantities, has made it possible to study details of fatty-acid formation from a particularly early stage of kernel development. This is of some importance as it has been possible to demonstrate that, although considerable changes in fatty-acid composition occur in very young material, a later increase in fat content (of some 400 per cent.) is accompanied by no change in the character of the oil. Simmons and Quackenbush (1954*a* and *b*) in their study of maturing soya beans obtained a very similar result, although soya-bean oil differs considerably in fatty-acid composition from that of palm kernels. Painter (1944) and Grindley (1950), using flax and cotton-seed respectively, both reported remarkably little alteration in oil character during maturation, although it is possible that any changes may have been completed before their earliest stage.

Mature palm kernels contain a most unusual mixture of fatty acids which is totally dissimilar to that in pericarp, root or leaf oils of the same plant (Boatman, unpublished), these last resembling fats from corresponding tissues of quite unrelated plants. An analysis by Carsten *et al.* (1945) of an impure testa fat shows that this too is of different fatty-acid content.

The immature endosperm contains oil of very different character from that of the mature nuts, and there appears to be a sudden physiological change in the tissue leading to the formation, in large quantities, of the new, highly saturated storage fat. It seems possible that in the youngest stages such oil as is present is basal protoplasmic fat and the low proportion found in these tissues would support this idea.

Most of the changes in fat composition which occur subsequently can be explained if it is assumed that this new highly saturated storage oil is laid down in addition to this original basal more unsaturated fat. No evidence has been found in this investigation to suggest that any interconversions of saturated and unsaturated acids occur during fat formation, although oleic acid shows somewhat anomalous behaviour, constituting a greater proportion of the total fatty acids in the oil from intermediate stages than in that from either younger or older. There is, however, no decrease in this acid at any phase examined, although it is interesting to note that Simmons and Quackenbush in their study of ripening soya beans have shown that radioactivity appeared first in oleic acid when plants were fed with marked sucrose. The fatty acid composition remains instead remarkably constant during deposition of large amounts of fat.

It has also been shown that, in this material, free fatty acids do not accumulate during active fat formation, and that evidence on this point from acid values is untrustworthy.

It seems doubtful if any great significance should be attributed to the fact that in oil-palm kernels and soya beans (Simmons and Quackenbush, loc. cit.) there is a progressive increase in saturation during ripening, whereas in flax

(Ivanow, 1911; Eyre, 1931; and Johnson, 1932) and *Macadamia* (Jones and Shaw, 1943) a corresponding decrease was noted, since this might be expected from the differences in character of the respective mature oils. Little is known at present about causes of these interesting variations.

ACKNOWLEDGEMENTS

I am most grateful to Mr. Toovey, the Director, and to Mr. Purvis, the Geneticist, of the West African Institute for Oil Palm Research, for the gifts of carefully selected material which have made it possible to carry out this investigation.

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The Effects of Changes in the Inorganic Nitrogen Supply on the Growth and Development of *Marsilea* in Aseptic Culture

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Received 21 July 1955

SUMMARY

The effects of changes in the inorganic nitrogen source of the medium on the growth and development of *Marsilea* in aseptic culture are described. A change in nitrate concentration to 0.2 or $5\times$ that of the normal medium had initially no effect on growth or heteroblastic development, but in the 0.2 nitrogen medium there was finally a reversion to juvenile stages followed by early cessation of growth.

Growth and heteroblastic development were stimulated by the substitution of the nitrate of the medium by ammonium salts or urea. Nitrite was little inferior to nitrate as a nitrogen source, but hydroxylamine was toxic even at low concentrations. Addition of sodium chloride led to improved growth and development in nitrate media, an effect referred to beneficial effects of the chloride ion.

Symptoms of toxicity and eventual death of the cultures which occurred after prolonged growth on ammonium media were due to an acid drift of the media rather than to a specific effect of the ammonium ion.

INTRODUCTION

FROM previous investigations on growth and morphogenesis in *Marsilea* (Allsopp, 1952, 1953, 1953*a*, 1954*a*, 1955) it was concluded (Allsopp, 1954) that the course of heteroblastic development is determined by the increase in size of the shoot apex, which in turn may be limited by the supply of nutrients necessary for protein synthesis. The evidence in support of this hypothesis was obtained principally by the study of the effects of changes in the carbohydrate supply. In the present work, the effects of changes in the inorganic nitrogen supply have been investigated. Apart from the immediate problem of heteroblastic development, such a study seemed of interest, since there has been comparatively little work on the nitrogen nutrition of intact vascular plants when growing under heterotrophic conditions.

MATERIAL AND METHODS

The aseptic sporelings of *Marsilea Drummondii*, A.Br., which were used throughout the present work, were obtained by the methods described previously (Allsopp, 1952).

In the majority of the experiments, control cultures were grown on the half-strength Knop's solution, which had been adopted as the standard medium for the present series of investigations on *Marsilea*. Before dilution to half-strength this medium had the composition in g./l.: KNO_3 , 0.25; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; KH_2PO_4 , 0.25. A modified Berthelot's solution of trace elements, as used by Gautheret (1942) was added to all media. Each medium also contained 2 or 4 per cent. glucose (A.R.) as an organic carbon source.

In experiments in which it was required to change the amount or nature of the nitrogen source, modifications of the Knop's solution were adopted:

Modified Knop's A, contained the normal quantities of magnesium sulphate and potassium dihydrogen phosphate, but the two nitrates were omitted. Changes in nitrogen content were obtained by addition of varying amounts of calcium nitrate.

Modified Knop's B, used in the majority of the experiments, contained equivalent amounts of the chlorides of calcium and potassium in replacement of the corresponding nitrates. Various salts were then added as *N* sources, as required by the particular experiment. Most media were sterilized by autoclaving for 15–20 minutes at 110–120° C., but in some cases filtration through sintered glass was adopted. The sporelings were grown in 10 or 20 ml. lots of medium in $\frac{5}{8}$ or 1 inch glass tubes respectively.

The usual number of replicates was ten. A higher number might have been statistically more reliable but a limit was set by the need to compare a number of treatments using megaspores from a single sporocarp. Significance of data was checked by variance ratio tests, calculated or evaluated by inspection and followed, if non-significant, by Student's *t*-test for significance of difference between sample means.

EXPERIMENTAL RESULTS

1. *Effects of nitrate concentration.* At the outset of the present work it was considered desirable to determine the effects produced by changes in the nitrogen concentration of the medium. Since previous work had shown that there are considerable morphological differences between sporelings grown on 2 and on 4 per cent. glucose media (Allsopp, 1953*a*, 1954*a*) the effects of changes in the nitrate concentration were investigated at these two sugar levels.

Series MD 7/10. In this experiment four different inorganic media were used, each at 2 and 4 per cent. glucose concentrations. The inorganic media were: (a) ordinary Knop's solution as control, (b) Modified Knop's A+0.2×calcium nitrate, (c) Modified Knop's A+1×calcium nitrate, (d) Modified Knop's A+5×calcium nitrate. The solution containing 0.2×normal quantity of calcium nitrate was used in two different amounts, 10 ml. and 20 ml.

Some of the principal results of this experiment are set out in Table I. It is evident from the table that the course of heteroblastic development

was not materially affected by the changes in the inorganic constituents of the media, i.e. at neither of the sugar concentrations did a change in mineral medium lead to any significant difference in the number of the node at which the first adult (quadrifid) leaf appeared (M.I.A.L. = Mean insertion of first adult leaf). The results from the two sugar concentrations, however, reveal that whichever mineral medium is considered, the first adult leaf appeared at an earlier node in the higher sugar concentration. There was no appreciable effect of nitrate concentration on the land-/water-form ratio, all the plants in the 4 per cent. glucose media having the characteristics of land-forms, whereas the plants from the 2 per cent. glucose solutions were principally water-forms, with some tendency towards shortening of the internodes in the $5 \times$ nitrate medium.

TABLE I

Effects of Nitrate and Glucose Concentrations on Leaf Development of Marsilea

Culture medium	No. of cultures	Glucose concentration				
		2% (a)		4% (b)		
		No. of M.I.A.L. ¹	S.D.	No. of cultures	M.I.A.L.	S.D.
Ordinary Knop's (N×1)	5	11.40	1.84	4	7.00	2.92
Modified Knop's A (N×1) + calcium nitrate	5	11.00	1.79	5	7.40	1.02
„ (N×5)	3	10.00	1.63	2	5.50	0.50
„ (N×0.2 10 ml.)	6	9.25	1.77	5	6.20	1.60
„ (N×0.2 20 ml.)	8	11.00	1.94	5	7.80	0.98
Collected data	27	10.57	1.92	21	6.48	1.75

For differences (a)–(b), $t = 7.408$ for 46 degrees of freedom: probability less than 0.1%. Differences between mineral salt treatments not significant at the 5% level of t .

¹ M.I.A.L. = Mean insertion of first adult (4-fid) leaf.

The rates of leaf- and root-production in the different media were also studied. As with the heteroblastic development, differences between the two sugar concentrations outweighed those due to changes in the inorganic constituents of the media, for at all nitrate concentrations the number of leaves and roots produced was greater in 2 than in 4 per cent. glucose media.

Since the change of nitrate concentration in this experiment had involved a corresponding change in the concentration of the calcium ion, it was felt that the experiment should be repeated in a different way without any change in calcium concentration.

Series MD 10/6 B. In this experiment only 2 per cent. glucose was used and Modified Knop's B was the basic mineral medium. Sporelings were given seven different treatments: (a) modified Knop's B only; (b)+sodium nitrate at $5 \times$ the usual nitrate concentration of Knop's solution; (c)+sodium nitrate ($\times 1$); (d)+sodium nitrate ($\times 0.2$); (e)+sodium nitrate ($\times 1$)+sodium chloride ($\times 4$); (f)+sodium nitrate ($\times 0.2$)+sodium chloride ($\times 4.8$); (g)+ammonium sulphate ($\times 2$). The sodium chloride added in treatments

(e) and (f) was of the amount calculated to raise the osmotic pressure of the medium to that of medium (b) with $5\times$ the usual concentration of sodium nitrate.

The results of this experiment, some of which are set out in Fig. 1 and Table II, showed clearly that change in nitrate concentration, over the range

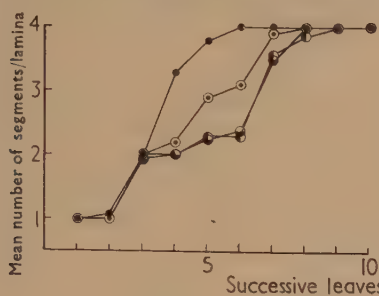


FIG. 1.

FIG. 1. Effects of sodium nitrate concentration and of sodium chloride on leaf segmentation.

$\text{NaNO}_3 \times 0.2$ —○, $\text{NaNO}_3 \times 5$ —●, $\text{NaNO}_3 \times 1 + \text{NaCl} \times 4$ —□,
 $\text{NaNO}_3 \times 0.2 + \text{NaCl} \times 4.8$ —●.

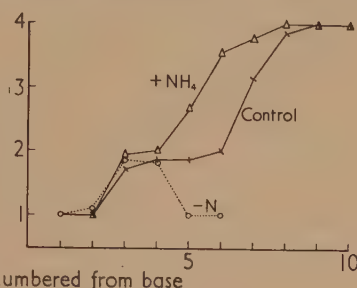


FIG. 2.

FIG. 2. Effects of ammonium sulphate and of medium without nitrogen on leaf segmentation.

TABLE II

Effects of Changes in the Medium on Leaf Production and Segmentation in *Marsilea*

Culture medium		Number of cultures	Leaf production after 5 weeks		Leaf production after 8 weeks		M.I.A.L.	S.D.
			Mean	S.D.	Mean	S.D.		
Modified Knop's B (N × O)		10	3.70	0.458	6.50	0.500
" + sodium nitrate (N × 5)		7	5.86	0.350	9.14	0.639	7.14	1.12
" + " (N × 1)		7	5.29	0.452	8.86	0.350	7.57	0.73
" + " (N × 0.2)		8	5.75	0.661	9.00	0.500	7.00	1.00
" + " (N × 1) + NaCl (× 4)		10	5.80	0.400	9.30	0.781	6.10	1.14
" + " (N × 0.2) + (NaCl × 4.8)		9	6.11	0.400	9.78	0.629	4.44	0.69
" + ammonium sulphate (N × 2)		9	7.89	0.567	8.78	0.987	6.00	0.94

employed, is without effect upon either the rate of leaf production or of heteroblastic development. In medium (a), with complete nitrogen starvation, growth of the sporelings was apparently normal during the formation of the first two or three leaves, but subsequent leaves were progressively smaller and showed reversion to a simpler leaf-type (Fig. 2). After prolonged exposure (some 6 months) a similar reversion was obtained in media (d) and (f), i.e. in those containing $0.2\times$ usual nitrate concentration. This reversion, which occurred while considerable amounts of glucose were still present in the medium, was evidently a consequence of exhaustion of the restricted nitrogen supplies. It was closely comparable with that previously described for sporelings in $0.2\times$ normal concentration Knop's solution (Allsopp, 1953) and, as in that experiment, occurred very abruptly.

The effects of the added sodium chloride are of interest. At both 0.2 and normal nitrate concentrations addition of sodium chloride led to stimulation of both leaf production and heteroblastic development; furthermore, the sporelings were more robust. It seems reasonable to conclude that the stimulation was due to the added chloride ion, and not to the sodium ion, for no appreciable difference in growth or development was observed between the plants growing in media (b) and (d) which differed in sodium nitrate concentration by 25 times.

2. *Effects of various inorganic nitrogen sources.* The results of the previous section indicated that uptake of nitrate from dilute solutions was so rapid that variation in the nitrate concentration of the medium was not a feasible method for altering the nitrogen nutrition of the sporelings. An alternative approach, however, was available in the use of various sources of nitrogen, which might be of different value to the plant.

Series MD 19/12. In this experiment seven different media were used as detailed in Table III. Results are set out in Table III and in Figs. 3 and 4.

During the first 6 weeks following inoculation the sporelings made considerably better growth in the ammonium—N media. The rate of leaf production was higher, as shown by Fig. 3. The heteroblastic development was also promoted, the adult leaf being attained at an earlier node (Table III and Fig. 4).

TABLE III

Effects of Changes in the Nitrogen Source on Leaf Production and Segmentation in Marsilea

Culture medium	No. of cultures	Leaf production after 9 weeks		M.I.A.L.	S.D.
		Mean	S.D.		
1a 2% glucose; Ordinary Knop's	9	12.67	1.25	11.44	2.59
1b 4% " " "	9	11.56	1.50	8.89	2.56
2 2% " " " + K and Ca chlorides (\equiv conc. in Modified Knop's B)	9	12.22	2.15	12.00	1.76
3a 2% glucose { Modified Knop's B	10	9.80	0.75	6.60	1.91
3b 4% " { + (NH ₄) ₂ SO ₄	9	9.22	1.03	7.78	1.23
4a 2% " { Modified Knop's B	10	11.10	1.45	7.30	1.19
4b 4% " { + NH ₄ NO ₃	8	11.38	2.00	4.13	0.33

After 6 weeks, signs of injury were evident in the newly formed organs of the plants in the media containing ammonium salts, and by the 11th week all these plants were dead, although those in the control media were still in active growth.

At the end of the experiment the pH in the control medium was close to the original value (5.7–6.0) but had fallen to 2.9–3.1 in the ammonium media. This pH was sufficiently low to kill the plants.

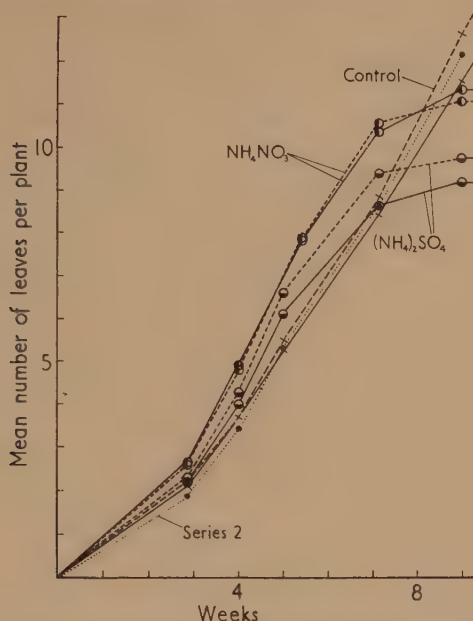


FIG. 3. Effects of nitrogen sources and glucose concentration on leaf production; broken lines, 2 per cent., continuous line, 4 per cent. glucose.

The absence of any toxic metabolic product in the ammonium media was confirmed by *Experiment MD 10/3*, in which the residual culture solutions from the treatment 3a of Table III were bulked and the pH adjusted to 6.2 by KOH solution. In all cases, plants continued active growth in this medium until drift to a low pH occurred.

Series MD 9/3. The two-fold aim of this experimental series was to provide some confirmation and clarification of the results of Series MD 19/12 and to test a number of other compounds as nitrogen sources for *Marsilea*.

Nitrogen was supplied throughout the present experiment at $0.2\times$ concentration of Knop's solution to reduce any possible toxic effects of too high a concentration of the various nitrogen sources employed. Modified Knop's B+4 per cent. glucose was used so that sugar concentration would not be a limiting factor.

Results with various nitrogen sources obtained are set out in Table IV and Fig. 5. It is clear from the figure, and from the M.I.A.L. results in the table, that the heteroblastic development was similar in the three ammonium media, and was completed earlier than in the nitrate or nitrite media. Under the conditions of the experiment, nitrite was almost as good a source of nitrogen as nitrate; the M.I.A.L. value was the same but the rate of leaf production somewhat lower. The results with urea were rather similar to those for ammonium nitrogen. In both the urea and ammonium media growth was vigorous initially but progressive root inhibition later set in, with

TABLE IV

Effects of Various Nitrogen Sources on Leaf Production and Segmentation in Marsilea

Substance tested	Sterilized by autoclaving (A) or filtering (F)	Survival of cultures	Leaf production after 4 weeks		Leaf production after 8 weeks		M.I.A.L.	S.D.
			Mean	S.D.	Mean	S.D.		
Sodium nitrate	A	7/8	4.50	0.76	10.83	1.46	6.00	1.73
"	F	3/7	4.0	0.82	10.0	1.41	6.33	2.05
Ammonium sulphate	A	4/9	5.0	0.71	7.75	1.65	4.00	0.71
" nitrate	A	10/10	5.0	1.61	10.40	2.54	4.50	1.12
" monohydrogen phosphate	A	6/7	4.17	0.91	7.67	1.16	4.17	0.90
Sodium nitrate	A	6/8	3.6	0.49	9.2	0.75	6.00	1.41
Urea	F	3/7	5.0	0.00	11.33	0.47	4.33	0.47
Hydroxylamine hydrochloride	A	0/6

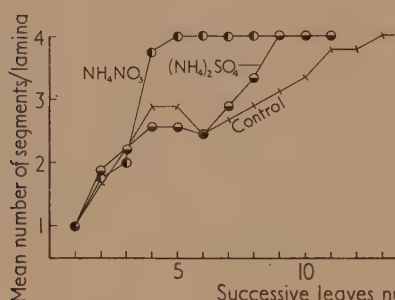


FIG. 4.

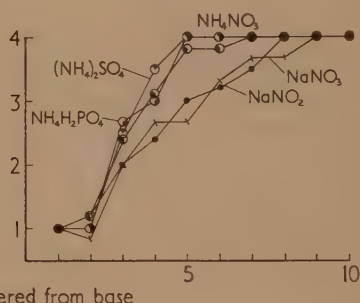


FIG. 5.

FIGS. 4 and 5. Effects of various nitrogen sources on leaf segmentation.

eventual death of the plants except in the ammonium nitrate medium. In the latter medium there was a progressive recovery from the earlier inhibition. This would be understandable if the ammonium ion were used preferentially, leading at first to a reduction of the pH to a toxic level, followed later by a rise in pH produced by utilization of the residual nitrate. Even at the low concentration employed, hydroxylamine was too toxic to serve as a nitrogen source.

Series MD 10/6 B. In this series ammonium sulphate was supplied at $2\times$ usual Knop's concentration. From Table II and Fig. 2, it is evident that growth and heteroblastic development were stimulated as in the other ammonium media already described.

DISCUSSION

One of the principal results of the present work is the observation that the heteroblastic development of *Marsilea* may be controlled by the nature and amount of the nitrogen supply. Under the conditions of the experiments, however, no change in heteroblastic development could be effected by a change in the concentration of the inorganic nitrogen component of the

medium. Such a result was perhaps only to be expected following the finding of Olsen (1953) that in the water-plant *Elodea* the uptake of ions is independent of their concentration in the external medium down to $3\mu\text{M}$.

In the present work, the absence of nitrogen from the medium prevented the attainment of the adult leaf. When nitrogen was present at $0.2\times$ normal concentration, there was no delay in heteroblastic development, but exhaustion of the nitrogen supply finally resulted in a reversion to juvenile leaves. A similar reversion had already been obtained in earlier work by carbohydrate starvation or by reduction in the concentration of the complete Knop's solution (Allsopp, 1953).

The results outlined above thus support the view advanced previously (Allsopp, 1954) that the heteroblastic development of *Marsilea*, and probably that of most other plants, is not normally limited by the synthesis of specific morphogenetic substances, but is dependent on the size and activity of the shoot apex, which in turn is controlled by the supply of the basic nutrients necessary for the synthesis of the various substances, particularly proteins, required for cellular growth and division.

Comparison of various nitrogen sources. If protein synthesis is indeed one of the principal processes limiting heteroblastic development, it might be expected that changes in the nature of the nitrogen supply would affect this process and consequently influence the course of development. Results of this kind were obtained, in fact, in several experiments. Thus it was shown that the adult quadrifid leaf appeared at an earlier node in media containing any of a number of ammonium salts than in media with nitrate or nitrite; a finding which is perhaps most simply explained by the assumption that ammonium is more readily assimilated than the nitrate or nitrite ion. In one of the few investigations carried out previously on the effects of mineral nutrients on the fern sporophyte, Schwabe (1953) found that leaf numbers of bracken plants were increased in his ammonium series and that the ammonium ion was taken up more rapidly than nitrate.

Effects of sodium chloride. The effects of the addition of sodium chloride to the medium also merit some consideration. As described in the experimental section, the presence of sodium chloride led to an increase in the rates of leaf and root formation and of heteroblastic development, and for the reasons stated the effect was ascribed to the chloride ion. Although the majority of workers on mineral nutrition have considered that chlorine is not an essential element, others have commented on the beneficial effects of added chloride (e.g. Miller, 1938; Lipman, 1938; Eaton, 1942; Tullin, 1954) while recently Broyer *et al.* (1954) have shown conclusively that in tomato, chlorine is an essential nutrient. The improved growth of *Marsilea* in the presence of chloride is thus readily understandable; but the underlying mechanism is less evident.

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Soluble Enzymes from Pea Mitochondria

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(Received 23 May 1955)

SUMMARY

A method of obtaining an extract of soluble enzymes from pea seedling mitochondria is described. Evidence is presented that the mitochondrial extract contains the following enzymes: Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) specific *isocitric* dehydrogenases, alcohol dehydrogenase, formic dehydrogenase, aldehyde dehydrogenase, glutamic dehydrogenase, malic enzyme, lactic dehydrogenase, fumarase, aconitase, DPN and TPN cytochrome-*c* reductases, adenylate kinase, phosphopyridine nucleotide transhydrogenase and oxaloacetic carboxylase. The relative activities of these enzymes have been quantitatively determined and the results discussed.

INTRODUCTION

DURING the course of experiments designed to demonstrate the presence of a diphosphopyridine nucleotide specific *isocitric* dehydrogenase in pea mitochondria (Davies, 1955), an extract of mitochondria was obtained, containing a number of soluble enzymes. This report deals with the assay of some of these enzymes and the demonstration of some enzymes not previously reported in higher plants.

METHODS

Chemicals. Diphosphopyridine nucleotide (DPN) 95 per cent. pure was prepared according to Kornberg and Pricer (1953), triphosphopyridine nucleotide (TPN) 85 per cent. according to Kornberg and Horecker (1953), cytochrome *c* (0.46 per cent. Fe) according to Margoliash (1954), adenosine triphosphate (ATP) according to Le Page (Umbreit, Burris, and Stauffer, 1949), crystalline alcohol dehydrogenase according to Racker (1950a), glucose-6-phosphate dehydrogenase according to Kornberg (1950), oxaloacetate according to Krampitz and Werkman (1941), *dl-isocitrate* according to Pucher and Vickery (1946). Coenzyme A (CoA) was a crude preparation isolated according to the method of Le Page and Mueller (1949) which assayed CoA 50 Lipmann units per mg. TPN 7 per cent, DPN 21 per cent. Reduced coenzyme A (CoASH) was prepared by incubating the crude CoA with a sulphhydryl compound, usually cysteine (100 μ M cysteine per 50 Lipmann units CoA) for 10 minutes prior to use. Reduced DPN was prepared by reduction of DPN using alcohol dehydrogenase, reduced TPN was prepared by reduction of TPN using glucose-6-phosphate dehydrogenase. Inosine

triphosphate (ITP) was supplied by Mr. R. Hems, *d*-isocitrate by Mr. D. Williamson. All other materials were obtained commercially.

Analytical. Spectrophotometric measurements were made at laboratory temperature (16–17°) with a Beckman DU spectrophotometer. DPN and TPN were determined by measuring the increased absorption at 340 m μ following enzymatic reduction with alcohol dehydrogenase and glucose-6-phosphate dehydrogenase respectively, using the extinction coefficient 6.22 \times 10⁶ cm.² mole⁻¹ (Horecker and Kornberg, 1948). The evolution of CO₂ during the decarboxylation of oxaloacetate was measured in Warburg manometers with conventional techniques. Protein was determined by the method of Warburg and Christian (1942).

Preparation of an extract of pea mitochondria. Mitochondria isolated from pea epicotyls (100 g.) by the method of Bonner and Millerd (1953), were suspended in 15 ml. 0.1 M. NaHCO₃ and disintegrated by shaking for 5 minutes with Chance Ballotini beads (size 14) in a Mickle shaker. The suspension was then frozen and thawed twice before being centrifuged at 24,500 \times g. in an International refrigerated centrifuge for 30 minutes. The supernatant was collected to give 12 ml. of a clear pale yellow extract. All operations were carried out at 0° C.

RESULTS AND DISCUSSION

Results of the assays for several enzymes are collected in Table I. The table is divided into groups according to the method of assay and it is convenient to discuss each group separately.

Group I. This group consists of enzymes determined by measurement of the reduction of DPN or TPN. Silica cells of light path 1 cm. were used containing, unless otherwise stated, tris(hydroxymethyl)-amino methane (THAM) buffer pH 7.4 (0.025 M.), DPN or TPN (0.6 μ moles), substrate (0.01 M.), and extract of pea mitochondria (0.2 ml.) in a total volume of 3 ml. (standard conditions.)

Tests for pyruvic and α -oxoglutaric dehydrogenases under the above conditions and also in the presence of CoA (100 Lipmann units), were negative. The enzymes found in the extract are listed in Table I.

DPN specific isocitric dehydrogenase has been shown to be unstable and to require a reducing agent for maximum activity (Davies, 1955). Consequently this enzyme was assayed immediately after preparation of the extract, by adding MnSO₄ (0.0015 M.) and cysteine (0.005 M.) to the standard reaction mixture.

TPN specific isocitric dehydrogenase is mainly located in the supernatant remaining after removal of mitochondria from a homogenate (Davies, 1954). To what extent the enzyme found in mitochondria represents adsorption from the supernatant remains uncertain.

Glutamic dehydrogenase has previously been demonstrated in plant tissues (Adler, Das, Euler, and Heyman, 1938; Berger and Avery, 1944), but its presence in plant mitochondria has not been reported.

TABLE I

Soluble Enzymes from Pea Mitochondria. Activities measured as μ moles product/hour/mg. protein during the first 10 minutes of the reaction at 16°.

Enzyme activity determined as in text unless otherwise stated.

Group	Enzyme	Activity	pH	Additions
1	DPN isocitric dehydrogenase . . .	0.42	7.4	MnSO ₄ (0.0015 M.) cysteine, (0.005 M.)
	TPN isocitric dehydrogenase . . .	0.37	7.4	MnSO ₄ (0.005 M.)
	Glutamic dehydrogenase . . .	0.48	7.4	
	Alcohol dehydrogenase . . .	0.20	9.1	
	Formic dehydrogenase . . .	0.44	7.4	
	Aldehyde dehydrogenase			
	(1) Acetaldehyde . . .	0.37	7.8	cysteine (100 μ moles)
	(2) Propionic aldehyde . . .	0.32	7.8	as above
	Malic enzyme . . .	0.32	7.4	MnSO ₄ (0.0015 M.)
	Lactic dehydrogenase . . .	0.98	7.4	
2	Fumarase . . .	3,000	7.4	
	Aconitase . . .	550	7.4	
3	DPN Cyt. <i>c</i> reductase . . .	600	7.4	
	TPN Cyt. <i>c</i> reductase . . .	400	7.4	
4	Adenylate kinase . . .	8.8	7.4	
	*Pyridine nucleotide transhydrogenase	0.28	7.4	
5	Oxaloacetic carboxylase			
	(1) ATP . . .	2.4	7.2	
	(2) ITP . . .	3.6	7.2	

* Activity removed by centrifuging for 30 minutes at 110,000 \times g.

Alcohol dehydrogenase has been studied in a number of plant tissues (Adler and Sreenivasaya, 1937; Davison, 1949a; Stafford and Vennesland, 1953). The enzyme activity was measured at pH 9.1 (THAM buffer 0.025 M.), because at pH 7.4 the equilibrium

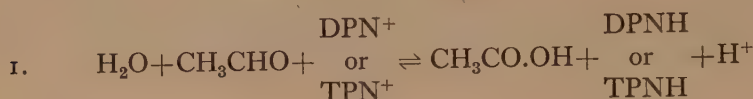
$$\frac{[\text{acetaldehyde}][\text{DPNH}][\text{H}^+]}{[\text{alcohol}][\text{DPN}^+]} = 1.15 \times 10^{-11}$$

is unfavourable for alcohol oxidation (Racker, 1950a).

Plant formic dehydrogenase has been investigated by Adler and Sreenivasaya (1937); Davison (1949b); and Mathews and Vennesland (1950), but its distribution between mitochondria and other cell fractions has not been investigated.

An aldehyde dehydrogenase which requires neither CoA nor DPN nor TPN, has been found in potato tubers (Franke and Schumann, 1942). The

aldehyde dehydrogenase reported here is a pyridine nucleotide linked enzyme (Fig. 1) and catalyses the reaction



The enzyme is unstable and attempts to purify it have been unsuccessful. Cysteine stimulates the activity of the enzyme (Fig. 2), suggesting that the

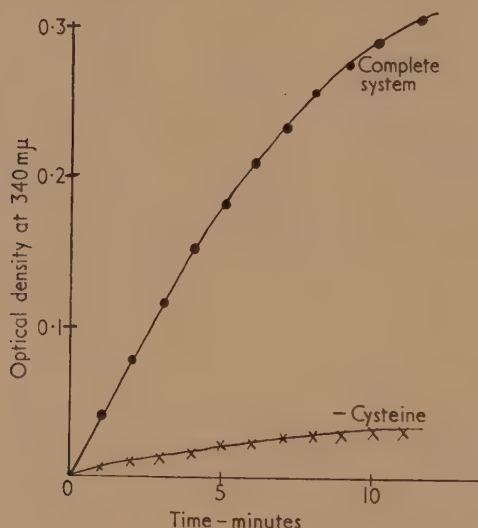
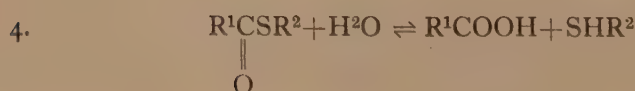
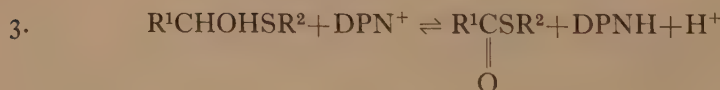


FIG. 1. Spectrophotometric demonstration of the reduction of DPN by acetaldehyde in the presence of an extract of pea mitochondria and cysteine.

Optical density at 340 mμ measured in silica cells light path 1 cm. containing acetaldehyde (50 μM), DPN (1 mg.), Cysteine (50 μM.) THAM buffer pH 7.8 (0.025 M.), extract 0.3 ml. in total volume of 3 ml. The blank contained all components except acetaldehyde.

enzyme is a thiohemiacetal dehydrogenase (Racker, 1955) catalysing the reactions



Mercaptoacetic acid, and to a lesser extent mercaptopropionic acid, could replace cysteine in the activation of the aldehyde dehydrogenase.

The malic enzyme of plants like that of animals is TPN specific (Vennesland and Conn, 1952). The activity was measured with TPN under standard conditions except that MnSO_4 (0.0015 M.) was added.

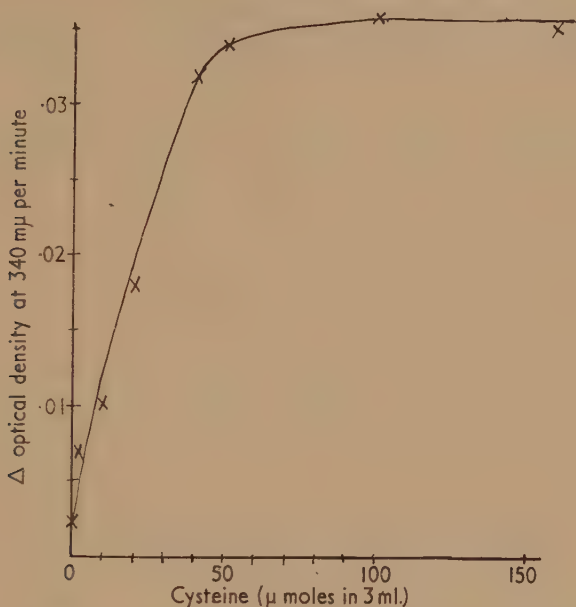


FIG. 2. Effect of cysteine on the reduction of DPN by acetaldehyde in the presence of an extract of pea mitochondria. Conditions as in Fig. 1 except that cysteine was varied as indicated.

Lactic dehydrogenase has been demonstrated in potato tubers (Barron, Link, Klein, and Michel, 1950) but Meeuse (1950) failed to detect this enzyme in pea seedlings. The presence of the enzyme in the mitochondrial extract was demonstrated by measuring the fall in optical density at $340\text{m}\mu$ when sodium pyruvate (10 μ moles) was added to cuvettes containing DPNH (0.4 μ moles), THAM buffer pH 7.4 (0.025 M.) and extract (0.2 ml.) in a total volume of 3 ml. The reduction of DPN (0.4 μ moles) by L-lactate (10 μ moles) was found to occur at a much slower rate.

Group II. Fumarase and aconitase were assayed according to the method of Racker (1950b). The increase in optical density at $240\text{m}\mu$, due to the double bond of fumarate and aconitate, was measured in silica cells of light path 1 cm., containing substrate (0.01 M.), THAM buffer (0.025 M.) and enzyme in a total volume of 3 ml. For the assay of aconitase the extract was activated with ferrous ammonium sulphate (5×10^{-4} M.) and cysteine (0.01 M.) (Morrison, 1954). The very high activity of fumarase and aconitase found deserves some comment. Krebs has pointed out that because of the high

activity of these enzymes, the fumarase and aconitase systems would be expected always to be at equilibrium and consequently there cannot be any change in free energy associated with these reactions. Thus in the reaction sequence succinate→fumarate→malate→oxaloacetate, if the fumarate/malate equilibrium is always maintained, the overall change of free energy can only occur in the first and last steps of the sequence.

Group III. DPN and TPN cytochrome *c* reductase activity was measured at a wavelength of 550 mμ in glass cells of light path 1 cm., containing DPNH or TPNH (0.3 μmoles), THAM buffer pH 7.4 (0.02 M.), oxidized cytochrome *c* (0.08 μmoles), and enzyme in a total volume of 3 ml.

Diaphorase activity was measured at a wavelength of 340 μm in silica cells of light path 1 cm., containing DPNH (0.3 μmoles), THAM buffer (pH 7.4, 0.02 M.), 2:6-dichlorophenolindophenol (0.125 μmoles), and enzyme in a total volume of 3 ml.

DPN and TPN cytochrome *c* reductases were very active (Table I). Both enzymes were stable and the properties of DPN cytochrome *c* reductase were studied in some detail.

Two flavoproteins have been isolated from heart muscle which are concerned in the oxidation of DPNH. One (DPN cytochrome *c* reductase) is a nonfluorescent flavoprotein, whose prosthetic group is a dinucleotide not identical with flavin adenine dinucleotide (FAD), and transfers electrons from DPNH to cytochrome *c* and certain dyes such as 2:6-dichlorophenolindophenol (Edelhoch, Hayaishi, and Tepley, 1952; Mahler, Sarkar, Vernon, and Alberty, 1952; Vernon, Mahler, and Sarkar, 1952). The other (diaphorase 1) is a fluorescent flavoprotein containing FAD as a prosthetic group and transfers electrons from DPNH to dyes but not to cytochrome *c* (Dewan and Green, 1938; Straub, 1939). Mahler and Elowe (1953, 1954) have recently demonstrated that DPN cytochrome *c* reductase, purified from pig heart, contains 4 atoms of iron per molecule of flavin. Removing the iron destroyed the capacity to react with cytochrome *c*, though diaphorase activity was retained.

Diaphorase 1 was demonstrated in pea seedlings by Lockhart (1939). Davison (1950) has demonstrated the presence of DPN cytochrome *c* reductase and diaphorase 1, also in pea seedlings.

Purification of DPN cytochrome c reductase.

(1) *First ammonium sulphate precipitation.* 8 ml. potassium phosphate buffer (0.5 M., pH 8.0) was added to 80 ml. of an extract of pea mitochondria, then solid ammonium sulphate (26.5 g.) was added with continuous stirring. After standing for 15 minutes the precipitate was removed by centrifuging (24,500 × g. for 10 minutes) and discarded. Ammonium sulphate (22 g.) was dissolved in the supernatant and after standing 10 minutes the precipitate was collected by centrifuging (24,500 × g. for 10 minutes), dissolved in water, the pH adjusted to 6 with acetate buffer (2.0 M. pH 5.7) and the volume made up to 20 ml. with water.

(2) *Second ammonium sulphate precipitation.* 5 minutes after the addition of ammonium sulphate (5.8 g.) the precipitate was removed ($24,500 \times \text{g.}$ for 10 minutes). Further ammonium sulphate (2.6 g.) was dissolved in the supernatant and after 5 minutes the precipitate was collected ($24,500 \times \text{g.}$ for 20 minutes) and dissolved in 8 ml. water.

(3) *Precipitation of inactive material at pH 4.9.* The enzyme solution was adjusted to pH 4.9 (measured with a glass electrode) by the addition of acetate buffer (pH 4.7, 0.5 M.). After 30 minutes the precipitate was removed

TABLE II

Purification of DPN cytochrome c reductase. Reductase assayed as in text. 1 unit of reductase equals optical density change of 0.1 per minute. Specific activity units per mg. protein

Step	Total DPN cyt. c. reductase activity	Soluble protein mg.	Specific activity DPN cyt. c. reductase	% Recovery	Purifica- tion
Extract	4,270	2,134	2.0	100	..
1st $(\text{NH}_4)_2\text{SO}_4$ ppt.	2,980	368	8.1	70	4
2nd $(\text{NH}_4)_2\text{SO}_4$ ppt.	820	20	41	19	20
Precipitation at pH 4.9	820	13.1	61	18.7	30
3rd $(\text{NH}_4)_2\text{SO}_4$ ppt.	220	2.5	88	5	44

($24,500 \times \text{g.}$ for 30 minutes) and the supernatant adjusted to pH 7.4 by the addition of THAM buffer (0.2 M. pH 8.0) and made up to 10 ml. with THAM buffer (pH 7.4).

(4) *Third ammonium sulphate precipitation.* Ten minutes after the addition of ammonium sulphate (3.5 g.) the precipitate was removed ($24,500 \times \text{g.}$ for 20 minutes). Further ammonium sulphate (0.5 g.) was dissolved in the supernatant and after 5 minutes the precipitate collected ($24,500 \times \text{g.}$ for 10 minutes) and dissolved in 5 ml. THAM buffer (0.02 M., pH 7.0).

The temperature throughout the purification was maintained at or below 0°C. The purification achieved is shown in Table II.

Properties of the purified enzyme.

pH activity curves. The effect of pH on the activity of diaphorase and cytochrome c reductase is shown in Fig. 3. Both enzymes show a maximum near pH 6.0 and the fall towards the alkaline range has the same trend in both cases. It was not possible to measure diaphorase activity with 2:6-dichlorophenolindophenol below pH 6 because the dye changes colour.

Relationship between diaphorase I activity and DPN cytochrome c reductase activity. The ratio

$$\frac{\text{DPN cytochrome c reductase activity}}{\text{diaphorase I activity}}$$

remains constant over a 40-fold purification (Table III), and this also applies to the discarded fractions. This suggests that both activities are due to a

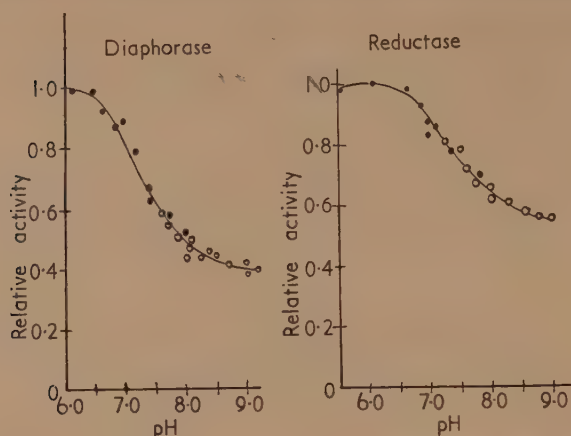


FIG. 3. Effect of pH on the activity of diaphorase and DPN cytochrome *c* reductase. Cell contents as in assay system except pH varied as indicated. ●—● phosphate buffer (0.02 M.) ○—○ THAM buffer (0.02 M.). Diaphorase activity measured by decrease in optical density at 600 m μ . DPN cytochrome *c* reductase activity measured by increase in optical density at 550 m μ . Enzyme specific activity 80.

single enzyme. Mathews (1951), however, obtained an enzyme from pea seeds which catalysed the oxidation of reduced DPN by methylene blue, but not by cytochrome *c*. The situation with respect to peas is thus similar to that found in animal tissues where diaphorase I has been purified by Straub (1939) and a cytochrome *c* reductase with diaphorase activity has been purified by Mahler, Sarkar, Vernon, and Alberty (1952).

TABLE III

Relationship between diaphorase I and DPN Cyt. c. reductase. Diaphorase and reductase assayed as in text. 1 unit equals optical density change of 0.1 per minute. Specific activity equals units per mg. protein

Step	Total diaphorase activity	Total reductase activity	Specific activity diaphorase	Specific activity reductase	Soluble protein mg.	% recovery	Purification	Ratio reductase activity diaphorase activity
Extract 60 ml.	1,080	3,625	0.51	1.71	2,120	100	0	3.3
ppt. A 0-18g. (NH ₄) ₂ SO ₄	270	820	0.56	1.7	482	25	0	3.0
" B 18-33g. " "	624	1,930	5.2	16.0	120	58	10	3.0
Refractionation of B at pH 6.0 (20 ml.) ppt C 0-5.8g. (NH ₄) ₂ SO ₄	55	175	2.3	7.2	24	5	4	3.1
Refractionation of B at pH 6.0 (20 ml.) ppt D 5.8-8.4g. (NH ₄) ₂ SO ₄	290	987	9.7	33	30	27	19	3.4
Refractionation of B at pH 6.0 (20 ml.) ppt E 8.4-10.4g. (NH ₄) ₂ SO ₄	160	502	4.1	12.5	40	15	9	3.0
Precipitation of inactive protein from E at pH 4.9 to give F	290	937	19.0	62.6	15	27	38	3.3
Refractionation of a fraction 'F' of lower activity than above pH; 7.4 vol. 10 ml.	250	840	11.0	37	23	3.4
ppt H 3-3.5g. (NH ₄) ₂ SO ₄	55	165	13.0	41	4	3.1
ppt I 3.5-4.0g. " "	75	227	20.7	68.8	3.6	3.3
ppt J 4.0-5.0g. " "	50	152	9.1	27.6	5.5	3.0

The reductase from peas was not inhibited by $\alpha:\alpha'$ -bipyridyl or by *o*-phenanthroline. However, after incubation with $\alpha:\alpha'$ -bipyridyl and DPNH for 12 hours at 0° C., or dialysis against 8-hydroxyquinoline for 12 hours, the DPN cytochrome *c* reductase activity was reduced but was not restored by the addition of ferric chloride (10^{-5} to 10^{-3} M.) (Table IV).

TABLE IV

Effect of chelating agents on diaphorase and reductase activity. Activity measured as in text. For experiments with $\alpha:\alpha'$ -bipyridyl enzyme specific activity 48, with 8-hydroxyquinoline 62.6

Treatment	Ratio Reductase activity diaphorase activity
1. None	3.1
2. Storage at 0° for 12 hours	3.1
3. Storage at 0° with $\alpha:\alpha'$ bipyridyl (0.002 M.) for 12 hours.	3.0
4. Storage at 0° with $\alpha:\alpha'$ bipyridyl (0.002) and DPNH (0.01 M.) for 12 hours	1.9
5. Storage at 0° with DPN (0.01 M.) for 12 hours	3.0
6. None	3.1
7. Dialysis for 14 hours against 0.02 M THAM pH 7.4	3.1
8. Dialysis for 12 hours against 8-hydroxyquinoline (saturated solution in 0.02 M THAM pH 7.4) and 2 hours dialysis against 0.02 M THAM pH 7.4	2.0
9. As 8 but Fe (10^{-3} M.) added after dialysis	2.3

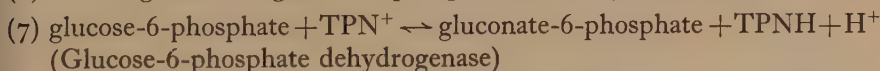
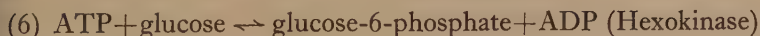
The Michaelis constants for DPNH (K_m^{DPNH}) were determined and the values

$$K_m^{\text{DPNH}} \text{ for diaphorase} = 7 \times 10^{-4} \text{ M.}$$

$$K_m^{\text{DPNH}} \text{ for reductase} = 5.4 \times 10^{-5} \text{ M.}$$

obtained at pH 7.4. If reductase and diaphorase activities involve a single enzyme and K_m^{DPNH} represents a simple equilibrium constant (Michaelis and Menten, 1913), then K_m^{DPNH} for diaphorase and reductase should be equal since the same equilibrium is involved in both reactions. It follows that either K_m^{DPNH} is not a simple equilibrium constant as suggested by Mathews but rather an equilibrium value involving a velocity constant for the acceptor molecule (Briggs and Haldane, 1925), or that the two activities involve separate enzymes.

Group IV. This group consists of enzymes determined by coupling with other reactions leading to the reduction of a pyridine nucleotide. The assay method for adenylate kinase (Kornberg and Pricer, 1951) depends on the reactions



Thus adenylate kinase may be assayed by observing the reduction of TPN in

a mixture containing the above components and in which adenylate kinase is the rate limiting step. The ADP sample contained a small amount of ATP which produced the initial rise in optical density seen in curve 2 (Fig. 4). The extract of mitochondria also contained a small amount of substrate which reacted in the assay system and produced the initial rise in optical density seen in curve 1 (Fig. 4).

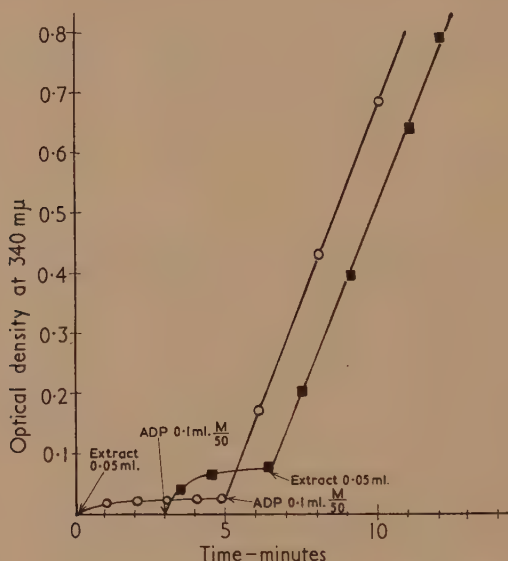


FIG. 4. Adenylate kinase activity of an extract of pea mitochondria. Silica cells contained THAM buffer (0.05 M.) pH 7.4, MgCl_2 (0.02 M.), glucose (20 μM .), TPN (1 μM .), hexokinase (0.1 ml. containing 5 mg. protein/ml.) and glucose-6-phosphate dehydrogenase (0.2 ml. containing 10 mg. protein/ml.) in a total volume of 3 ml. ADP (0.1 ml. M./50) and extract of mitochondria (0.05 ml.) added as indicated.

Adenylate kinase (myokinase) was first discovered in muscle (Colowick and Kalckar, 1943) and shown to be relatively stable to boiling in 0.1 N HCl (21 per cent. loss of activity after 10 minutes). Subsequently an adenylate kinase was discovered in liver mitochondria (Kotelnikova, 1949; Barkulis and Lehninger, 1951; Kielly and Kielly, 1951; Novikoff, Hecht, Podber, and Ryan, 1952), which is heat labile, 95 per cent. of the activity being lost at 90° C. in 1 minute in 0.05 N HCl and also at neutral pH (Novikoff *et al.*, 1952). Leuthardt and Brutin (1952) found an adenylate kinase in the soluble part of liver homogenates which resembled the enzyme described by Colowick and Kalckar in being stable to boiling in 0.1 N HCl. The enzyme from pea seedling mitochondria is also relatively heat stable under acid conditions, but is heat labile at pH 8.0 (Table V). The pH activity curve of the enzyme is shown in Fig. 5. Each activity was determined at two or more concentrations of hexokinase and glucose-6-phosphate dehydrogenase to ensure that adenylate kinase was the rate limiting reaction.

TABLE V

Stability of adenylate kinase isolated from pea mitochondria. Cell contents as in Fig. 5, except pH maintained at 7.5 with THAM buffer (0.05 M). Activity measured by increase in optical density at 340 m μ during the first 3 minutes.

Activity of adenylate kinase after heating
under conditions specified below

Time of heating	50° C.	90° C.	
	pH 8.0 THAM buffer (0.005 M.)	pH 8.0 THAM buffer (0.005 M.)	90° C. 0.05 N HCl.
0	0.150	0.150	0.150
2	0.097	0.074	0.140
5	0.061	0.057	0.131
10	0.04	0.043	0.128
15	0.023	0.014	0.101

The level of adenylate kinase activity was high in relation to the rate at which ATP is generated from ADP.

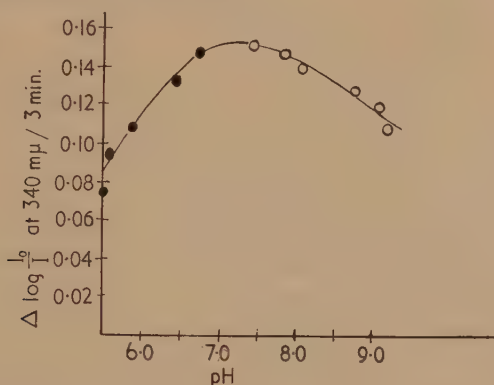
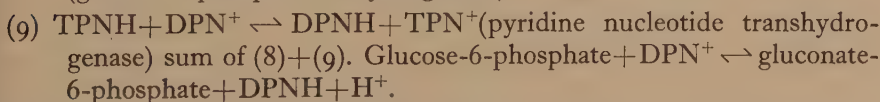
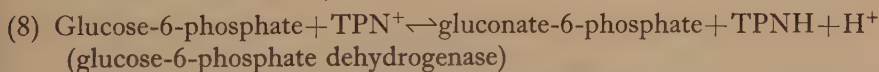


FIG. 5. Effect of pH on the activity of adenylate kinase. Cell contents as in Fig. 5 except TPN (0.02 μ M.), AD P (0.5 μ M.), extract of mitochondria diluted 5-fold and pH varied as indicated. ● succinate buffer (0.05 M.). ○ THAM buffer (0.05 M.). Activity measured by increase in optical density at 340 m μ during the first 3 min.

Pyridine nucleotide transhydrogenase (Kaplan, Colowick, and Neufeld, 1953) was assayed by adding the extract to a reaction mixture containing glucose-6-phosphate, glucose-6-phosphate dehydrogenase, DPN, and a trace of TPN.

The reactions involved are,



The formation of reduced DPN was confirmed by the addition of acetaldehyde and alcohol dehydrogenase which is specific for DPN (Fig. 6). The

relative activity of this enzyme was found to be of the same order of magnitude as the dehydrogenases. The biological significance of this enzyme is not clear, though as suggested by Kaplan *et al.* (1953), the enzyme may promote an exchange of hydrogen between bound DPN and free DPNH.

Group V. Oxaloacetic carboxylase (Utter and Kurahashi, 1953) was assayed by measuring the rate of decarboxylation of oxaloacetate by the extract, in the presence and absence of ATP and ITP, the extra CO_2 being a measure

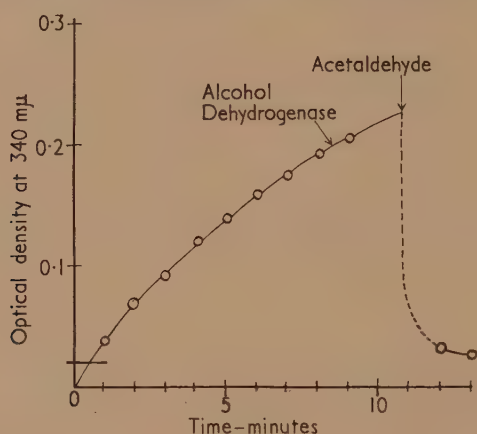
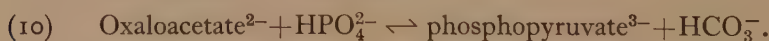


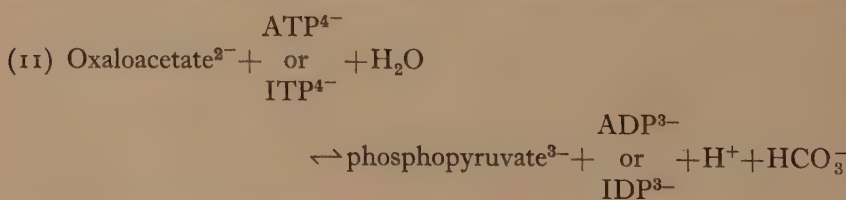
FIG. 6. Spectrophotometric evidence for the presence of pyridine nucleotide transhydrogenase in extracts of pea mitochondria. Cells contained glucose-6-phosphate ($5 \mu\text{M.}$), TPN ($0.01 \mu\text{M.}$), DPN ($1 \mu\text{M.}$), glucose-6-phosphate dehydrogenase (0.2 ml. containing 10 mg./ml.) THAM buffer pH 7.4 (0.02 M.), extract (0.3 ml.) in a total volume of 3 ml. Acetaldehyde ($50 \mu\text{M.}$) and alcohol dehydrogenase (1 mg.) added as indicated.

of the activity of oxaloacetic carboxylase (Fig. 7). The formation of phosphopyruvate was confirmed by isolating the phosphopyruvate by filter paper chromatography (Bartley, 1954).

An enzyme present in acetone powders of spinach leaves (Bandurski and Greiner, 1953) carries out the reaction.



This enzyme does not require ADP and therefore differs from the enzyme described by Utter and Kurahashi (1954) which catalyses the reaction



An ATP catalysed decarboxylation of oxaloacetate, which Bartley (1954)

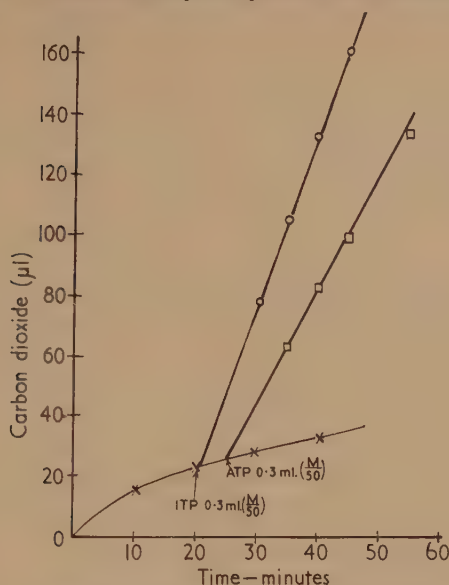
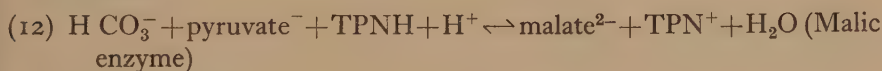


FIG. 7. Effect of ATP and ITP on the decarboxylation of oxaloacetate in the presence of an extract of pea mitochondria. Flasks contained oxaloacetate ($80\mu\text{M}$), glutathione ($5\mu\text{M}$), MnCl_2 ($1\mu\text{M}$), phosphate buffer pH 7.2 (0.05 M), extract (0.5 ml) in a total volume 2 ml . ATP and ITP added as indicated. $95\%\text{N}_2$ $5\%\text{CO}_2$ in the gas space, bath temperature 25° .

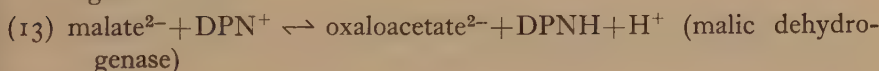
has shown to be present in animal mitochondria, is also present in pea mitochondria (Table I).

The formation of phosphoenolpyruvate may have a role in the metabolism of dicarboxylic acids. Succulent plants fix CO_2 in malic acid during darkness (Bonner and Bonner, 1948; Thomas, 1949) and in light there is a release of CO_2 and a disappearance of malate. It is suggested that during the dark period the fixation of CO_2 in malate provides a means of circumventing the energy barrier involved in the synthesis of phosphopyruvate from pyruvate (Krebs, 1954). In light phosphopyruvate is formed from malate by reactions (13) and (14).

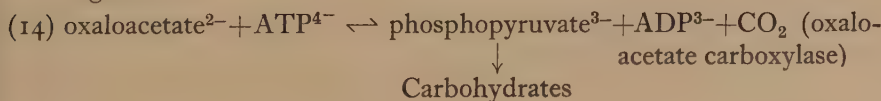
In darkness



In light and dark



In light



These reactions do not involve a gain in carbon and are not directly related to photosynthesis. It is, however, possible that these reactions are involved in the sudden outburst of CO_2 which occurs when *Chlorella* is illuminated after a period in darkness (Emerson and Lewis, 1941) and also in the decarboxylation of malate by beet slices, observed by Bennet-Clark and Bexon (1943) who suggested that phosphopyruvate was formed from malate.

GENERAL DISCUSSION

The results demonstrate the presence in pea seedling mitochondria, of soluble enzymes concerned in some of the reactions of the tricarboxylic acid cycle and associated reactions, including electron transfer and phosphorylation. Pea mitochondria were found to contain the same enzymes, which previous authors have demonstrated in liver mitochondria.

The relative activities of the enzymes listed in Table I, though minimum values, show that the activities of the dehydrogenases are of the same order of magnitude as the rate of substrate oxidation by intact mitochondria. Thus isocitric-dehydrogenase activity was found to be $0.79 \mu\text{moles}$ of isocitrate oxidized per hour per mg. protein at 16° (Table I) and oxidation of tricarboxylic acid by intact pea mitochondria was found to be $0.6\text{--}0.8 \mu\text{moles}$ at 20° (unpublished results).

ACKNOWLEDGEMENTS

The author is indebted to Professor H. A. Krebs, F.R.S., and Dr. W. Bartley for their advice and criticism. This work was carried out during the tenure of an Agricultural Council Research Fellowship.

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Studies in Plant Growth Hormones

IV. CHROMATOGRAPHY OF HORMONES AND HORMONE PRECURSORS IN CABBAGE

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Received 21 October 1955

SUMMARY

1. Acid and neutral ethereal fractions and the non-ether-soluble aqueous fraction of an extract of cabbage leaves were chromatographed, and the chromatograms assayed using oat coleoptile sections.

2. In the aqueous fraction an acidic precursor of 3-indolylacetonitrile (IAN) was found. When chromatographed in *isopropanol/ammonia*, the precursor travelled at the position of 3-indolylacetic acid (IAA), but in *n*-butanol/ammonia it was much closer to the starting-line. IAN is liberated from the precursor under conditions of alkaline hydrolysis including ammoniacal chromatography, and is also liberated by heat. Precursor and IAN zones promoted coleoptile growth, but the former when sprayed with ferric chloride/perchloric acid or nitrous/nitric acid gave a yellow colour showing that there was no free IAN on this part of the chromatogram. Hypotheses to account for this activity are discussed.

3. A neutral inhibitor was present in the aqueous fraction. It is volatile, ether-soluble, and is thought to have been liberated from a water-soluble substance.

4. The neutral fraction, chromatographed in *isopropanol/ammonia*, contained IAN and a growth promoter at R_f 0.0-0.1: the latter stimulates cress root growth above that in water. This promoter could be formed from a precursor in the aqueous fraction by heat treatment followed by shaking with sodium bicarbonate solution. It is suggested that this neutral hormone is the accelerator- α of Bennet-Clark and Kefford. The data of these workers are analysed to show that this interpretation is consistent with their results.

5. The acid fraction contained IAN but no IAA. The former is thought not to have been liberated from the precursor in this fraction but to have entered into it from the neutral fraction during separation with sodium bicarbonate solution. Although IAA may have been absent from the plant material, it is possible that any present was destroyed during the process of extraction.

6. Evidence is presented that there are other growth promoters present at low concentration in the extract in addition to those already mentioned.

INTRODUCTION

In auxin studies it is frequently asserted that 3-indolylacetic acid (IAA) is either the dominant hormone or the only naturally occurring growth promoter in plants. Its biogenesis is unknown, but it is generally assumed to originate from tryptophane with 3-indolylimino acid, 3-indolylpyruvic acid

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(IPyA), tryptamine, and 3-indolylacetaldehyde as possible intermediaries (Larsen, 1951).

By the application of paper chromatography to the separation of growth regulators in plant extracts, Bennet-Clark and Kefford (1953) found that two and sometimes three zones of growth promotion were present on their chromatograms of the acid fraction of several plant shoot and root extracts. One zone was attributed to IAA, while another of smaller Rf value was assumed to result from a single substance, termed accelerator- α . Stowe and Thimann (1953 and 1954), after comparing chromatograms of synthetic IPyA with those of a maize seed extract, concluded that accelerator- α and IPyA are identical; however, other studies on IPyA at Manchester (Bentley, Farrar, Housley, Smith, and Taylor, 1956) show that it is broken down by chromatography under the conditions which Stowe and Thimann used to demonstrate its occurrence in maize. Thus, the presence of IPyA in plant material cannot be accepted before a more critical examination has been made.

The only growth promoter other than IAA isolated from vegetative tissues of a higher plant is 3-indolylacetonitrile (IAN) (Jones, Henbest, Smith, and Bentley, 1952). Although this substance is highly active in promoting growth of oat coleoptiles (Bentley and Housley, 1952 and 1953), it is inactive on pea stems (Bentley and Bickle, 1952; Thimann, 1953) and on maize coleoptiles (unpublished data from this laboratory). Stowe and Thimann (1954) have shown that IAN may be converted to IAA in oat coleoptiles under certain conditions and they believe that this conversion is necessary before growth activity can be shown. Gordon (1954) considers that the inability of certain plant organs to carry out an IAN-IAA conversion raises doubt as to the participation of IAN as an intermediary in auxin biogenesis; however, he concludes that there is insufficient information on its occurrence, origin, and mechanism of action to judge whether this is true.

It is of interest that the third zone of growth promotion on the chromatograms of Bennet-Clark and Kefford (loc. cit.) occurred at the Rf of IAN. With extracts of *Aegopodium* rhizome and potato tuber, they obtained an IAN chromogenic reaction with nitrous/nitric acid at this position, and to explain its occurrence in the acid fraction it was suggested that separation of acid and neutral material had been incomplete. An alternative explanation is afforded by the work of Bonde (1953), who obtained evidence of an acidic precursor of a neutral growth substance in cabbage; thus, it is possible that the presence of IAN in the acid fraction was not due to incomplete separation but to liberation from a precursor. Bonde found that his precursor appeared to be water-soluble rather than ether-soluble; this was of interest to us as it was consistent with unpublished Manchester data which showed that the water-soluble ether-insoluble fraction of a cabbage extract possesses auxin activity.

This paper reports the results of an investigation of the hormones and hormone precursors present in cabbage at maturity. Ethereal and aqueous fractions of an extract have been examined by paper chromatography and *Avena* straight-growth bioassay. Precursors were found in the aqueous frac-

tion, and these have been examined. The steps leading from one precursor to the formation of its hormone have been traced, and it is thought that the latter is identical with the accelerator- α of Bennet-Clark and Kefford (loc. cit.).

MATERIALS AND METHODS

Materials. All chemicals commercially supplied were of the purest grade obtainable. Anaesthetic ether was rendered and maintained peroxide-free by standing over sodium wire in darkness at 0° C. Single glass-distilled water was used for preparing all solutions. Victory oats and Holdfast wheat of the 1951 harvest were obtained from the National Institute of Agricultural Botany, Cambridge; cress seed was kindly supplied by Dr. Moewus of Sydney University, Australia.

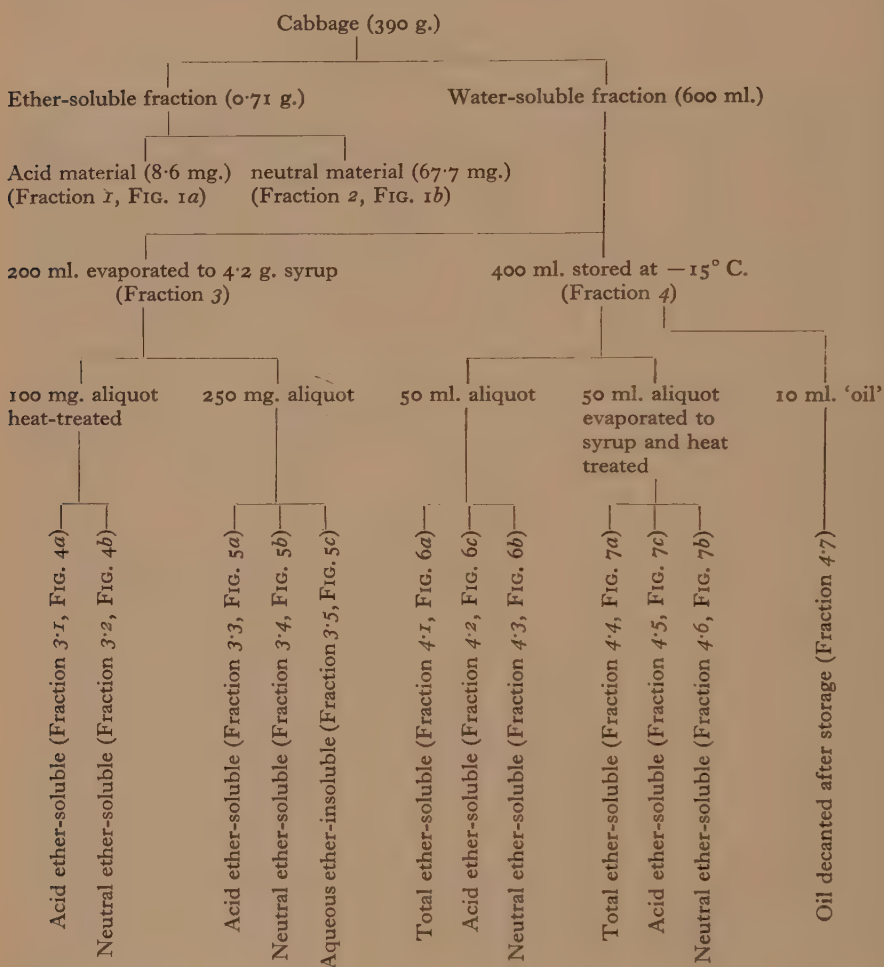
Extraction technique. A single plant of summer cabbage was collected in early October 1953. The inner blanched leaves (390 g.) were quickly frozen with solid carbon dioxide, and then ground to a coarse comminution. Extraction with periodical stirring took place in 1,500 ml. of a mixture of 95 per cent. ethyl alcohol and sulphuric acid (pH 3.2) for 40 hours at -10° C. During the extraction the pH repeatedly drifted and had to be restored by the addition of more acid.

The pH of the tissue-free extract was raised to 5 with barium hydroxide, the extract filtered, and the alcohol removed at 35° C. by distillation under reduced pressure. The pH of the aqueous remainder (600 ml.) was reduced to 3 by sulphuric acid and the solution thoroughly extracted four times with a total volume of 1,400 ml. ether. The ethereal and aqueous fractions were subsequently divided into sub-fractions as shown in the table below.

The ethereal fraction was washed in two parts, each with 200 ml. water, before being dried over anhydrous magnesium sulphate, filtered, and distilled to a dry extract (0.71 g.). This was redissolved in fresh ether and separated into acid and neutral fractions using sodium bicarbonate solution (Bentley and Housley, 1952). A thick emulsion was obtained which was extracted with an excess of fresh ether. The remaining emulsion was frozen and then allowed to thaw slowly; it separated completely into ethereal and aqueous layers. The combined ethereal solutions contained the neutral fraction, while the corresponding acid fraction was obtained from the acidified bicarbonate phase (pH 3) by extraction with ether. Both ethereal solutions were washed with 25 ml. water before being dried, filtered, and the filtrates taken to dryness to yield 8.6 mg. acid material (fraction 1) and 67.7 mg. neutral material (fraction 2).

The 600 ml. aqueous fraction was divided into two portions of 200 ml. and 400 ml. (fractions 3 and 4). The pH of fraction 4 was raised to 5 with barium hydroxide, the solution filtered, and then stored at -15° C. until required for future examination. Fraction 3 was extracted with 2×200 ml. ether to ensure that ether-soluble materials were fully removed. The pH was then raised to 5.3, the solution filtered, and the filtrate slowly concentrated under reduced

pressure at 25–26° C. (an accidental increase in pressure in the apparatus resulted in a rise in temperature to 40° C. for 2 minutes) yielding a thick brown syrup (4.2 g.) which was stored at -15° C.



Chromatography technique. Plant extracts were dissolved in a suitable solvent and placed on a Whatman No. 1 paper with a capillary pipette to form a continuous uniform starting-line, avoiding direct sunlight. Chromatograms were developed in the dark at room temperature without prior equilibration in either 4 vol. isopropanol + 1 vol. 0.15N. ammonia, or *n*-butanol saturated with 1.5N. ammonia. They were developed using the descending technique, until the solvent front was 30 cm. from the starting-line, dried in a current of air at room temperature for 20 minutes, and then examined in filtered U.V. light (2,537 Å transmitted) for fluorescence.

Chromatograms were usually divided for bioassay according to the fluores-

cence pattern: fluorescence which was useful for locating active zones is shown below histograms in certain figures.

Each division was eluted in 12 ml. water: chromatographic solvents were removed under reduced pressure with air drawn through the solution at a bath temperature of 35–40° C.

Spray reagents. Chromogenic reactions were developed by drying the papers at 35° C., and then heating at 50–60° C. for 1–5 minutes. The following sprays were used: ferric chloride/perchloric acid (2 ml. 0.05 M. FeCl_3 +100 ml. 5 per cent. HClO_4), nitrous/nitric acid (1 g. KNO_2 in 200 ml. HNO_3 S.G. 1.42 diluted $\times 10$), *p*-dimethylaminobenzaldehyde (2 g. in 20 ml. HCl (S.G. 1.18)+80 ml. absolute ethanol).

Bioassay techniques. The method of *Avena* straight-growth assay has been reported (Bentley, 1950), and the techniques used described in detail (Bentley and Housley, 1954). 10 mm. sections were cut from coleoptiles of either 1.4–1.6 cm., 1.6–1.8 cm., or 1.8–2.0 cm., floated on 10 ml. test solution in 2 inch Petri-dishes, and their length measured after approximately 17 hours. Ten replicates per treatment were used except when stated to the contrary.

The method of cress assay is a modification of that of Moewus (1949*a* and *b*). Seeds, placed 1 cm. apart, are germinated in Petri-dishes lined with wet filter paper. Twenty-four to 28 hours later, six seedlings with roots of 5 mm. length are placed into a 2-inch Petri-dish containing 10 ml. 1.5 per cent. agar into which the test solution has been previously incorporated. The roots grow into the agar, and approximately 17 hours later their length is determined to the nearest mm. by measuring on graph paper after removal from the agar medium. The assay is carried out at 27° C. in phototropically inactive light, and subsequent root growth is at 25° C. in the dark.

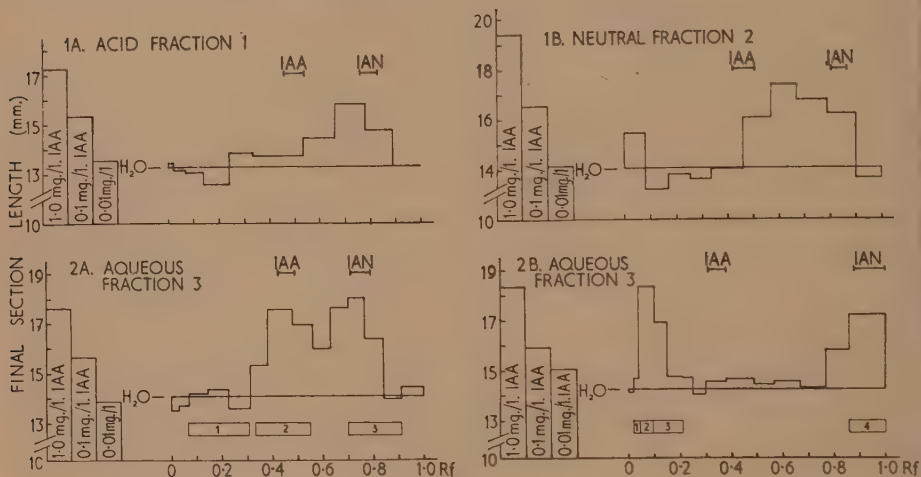
RESULTS

Chromatography of the acid and neutral ether-soluble fractions. Histograms of the acid fraction 1 (8.6 mg.) and neutral fraction 2 (10 mg.) after chromatography in *isopropanol/ammonia* are shown in Fig. 1. In fraction 1 growth promotion at R_f 0.54–0.89 lies closer to the expected position for IAN than IAA, while behind this slight promotion extends to R_f 0.24. There is no peak corresponding to IAA, and if it is present it can only be at low concentration. Likewise, it would appear that if accelerator- α is present, it too is at low concentration.

In the neutral fraction growth promotion extends from R_f 0.46 to 0.9; $\times 10$ to $\times 100$ dilutions of the solutions show that the main stimulation lies at the IAN position. As IAN has been isolated from cabbage (Jones *et. al.*, 1952), it is reasonable to attribute the growth to this hormone. Nearer the starting-line stimulation occurs at R_f 0–0.085, but at $\times 10$ dilution it is barely significant.

Chromatography of the aqueous fraction. Chromatography of the syrup of fraction 3 (approximately 50 mg.), was carried out initially in *isopropanol/ammonia*: a typical result is shown in Fig. 2*a*. Peaks of growth promotion are

shown at IAA and IAN regions: chromogenic sprays gave shades of yellow with ferric chloride/perchloric acid and nitrous/nitric acid between Rf 0.1 and 0.5; near IAN, nitrous/nitric acid produced a pale mauve colour resembling that of IAN at low concentration.



FIGS. 1 and 2. Chromatography in ammoniacal *isopropanol* of the acid and neutral ether-soluble fractions 1 and 2 of an extract of cabbage leaves (1a, 1b). Chromatography of the aqueous fraction 3 of an extract of cabbage leaves in ammoniacal *isopropanol* (2a) and *n*-butanol (2b). U.V. fluorescence on the chromatograms is shown below. 2a:—zone 1, yellow pigment on blue background; 2, blue with faint purple; 3, bluish-purple. 2b:—zone 1, light blue; 2, purple; 3, bluish-purple; 4, bluish-purple.

The demonstration of a growth promoter at the IAA position but not giving a red chromogenic reaction typical of IAA prompted the examination of the syrup in another solvent system, *n*-butanol/ammonia. The result (Fig. 2b) differs from that of Fig. 2a. Activity no longer corresponds to IAA, but instead growth promotion is close to the starting-line; activity also occurs, at the IAN position. Results with chromogenic sprays gave yellow at Rf 0.0–0.2 and mauve at the IAN position.

These results, in which the pattern of growth promotion varies with the solvent system used, suggested that the growth-active zones at the IAA position (Fig. 2a) and at the starting-line (Fig. 2b) should be examined further to see whether they are identical.

Two 28 cm. chromatograms were each loaded with 200 mg. syrup and chromatographed in *isopropanol*/ammonia. U.V.-fluorescent zones corresponding to 1 and 2 in Fig. 2a were removed and eluted at 5° C. with 4 ml. water. 0.75 ml. of this eluate was pipetted on each of two papers, and chromatographed in ammoniacal *isopropanol* or *n*-butanol (Fig. 3a and b). The histogram of the *isopropanol* chromatogram is essentially identical with that of Fig. 2a, while that in *n*-butanol differs but slightly from that of Fig. 2b. In Fig. 3b, cutting of the chromatogram at Rf 0.0–0.08 was based on U.V.

fluorescence, but it is too uneven to enable one to conclude that two growth promoters have been separated. Before drawing conclusions from this experiment, data of the complementary experiment are presented.

Two chromatogram papers were prepared as in the preceding experiment and chromatographed in *n*-butanol ammonia. Two zones were removed, the area of initial loading and the following zone to R_f 0.18 (corresponding to U.V.-fluorescent zones 1–3 in Fig. 2*b*). These were eluted with 1 ml. and 2 ml. water respectively. 0.4 ml. of each eluate was rechromatographed in ammoniacal isopropanol or *n*-butanol. The histogram of the starting-line eluate in isopropanol ammonia (Fig. 3*c*) resembles Fig. 2*a* except for differences in relative position of the marker spot of IAN and the growth promoter near the solvent front. In *n*-butanol ammonia (Fig. 3*d*) the histogram is basically similar to Fig. 2*b*, but there is an ill-defined peak at R_f 0.8 and continuous tailing behind it. It is probable that growth promotion at R_f 0.06–0.19 does not actually overlap the IAA control but that division of the chromatogram has caused this effect.

Histograms of the remaining eluate differ slightly from those described above. Chromatography in isopropanol/ammonia (Fig. 3*e*) has produced appreciable growth at R_f 0.12–0.25, and the same material may yield the zone at R_f 0–0.02 on the *n*-butanol chromatogram (Fig. 3*f*); however, uneven chromatogram division prevents one from stating this with certainty.

The results of Fig. 3 indicate that the substance at the IAA zone on isopropanol chromatograms is identical with that near the starting-line on *n*-butanol chromatograms. Thus, differences in relative position of this substance and IAA result from chromatographic factors, and not from chemical changes due to the different solvent systems. From Fig. 2 it is clear that chromatography in *n*-butanol gives better separation of active zones, which in this solvent may be complete. One may deduce from Fig. 3*c–f* that promotion in the IAN region originates from a precursor on and near the starting-line of *n*-butanol chromatograms; in isopropanol this precursor occurs at the IAA zone and/or in a zone of smaller R_f range (see Fig. 3*a* and *b*).

Comparison of Fig. 3*c*, 3*e*, and 2*a* suggests that there is a growth promoter at R_f 0.1–0.2, but that it is probably being formed from another substance. This suggestion would seem to be confirmed by the lack of the peak in Fig. 3*a*. It is possible that unstable compounds are involved, and small differences in technique are influencing their reactions.

Chromatography of ether extracts of the aqueous fraction. It has been shown that hormones and their precursors are present in the aqueous fraction.

Although these substances are essentially water-soluble, it is probable that they are slightly ether-soluble too. Therefore, it will probably be possible to extract with ether, separate the acid substances by a partition with sodium bicarbonate, and detect their physiological activity after chromatography.

In preliminary experiments it was noticed that heat treatment of the syrup of fraction 3 increased the amount of activity on chromatograms at the IAN zone, and that the substance in this zone appeared to be ether-soluble; there-

fore, to examine this growth promoter more fully, the following experiment was first carried out. 100 mg. of fraction 3 syrup were heated at 98–100° C. for 25 minutes, cooled, dissolved in 20 ml. sodium bicarbonate solution, extracted with 3 × 25 ml. ether, and the ethereal phase reduced to a small volume (fraction 3.2). The acid fraction 3.1 was obtained by acidifying the aqueous phase to pH 3 with sulphuric acid, extracting with the same volume of ether, and then concentrating in a similar manner. Chromatography was carried out in *isopropanol/ammonia*.

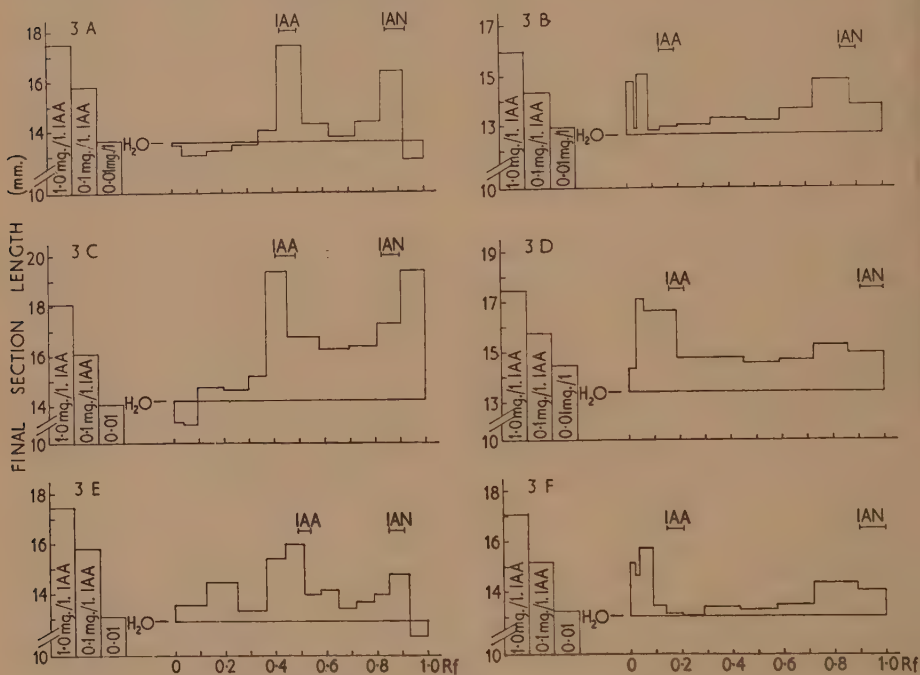


FIG. 3. Chromatography of fraction 3 of an extract of cabbage leaves. 3a and b:—Chromatography was first carried out in ammoniacal *isopropanol*. The U.V.-fluorescent zones corresponding to 1 and 2 in Fig. 2a were eluted with water and rechromatographed in ammoniacal *isopropanol* (3a) and *n*-butanol (3b). 3c-f:—Chromatography was first carried out in ammoniacal *n*-butanol. The starting-line was eluted in water and rechromatographed in ammoniacal *isopropanol* (3c) and *n*-butanol (3d). Following this zone, U.V.-fluorescent zones corresponding to 2 and 3 in Fig. 2b were treated in a similar manner (3e and f respectively).

In the neutral fraction (Fig. 4b) the greatest activity occurred at the IAN zone. U.V. fluorescence at this position was identical in appearance with that of IAN, while R_f limits were essentially similar too. On spraying with nitrous/nitric acid a purplish-blue colour appeared which changed with continued heating to an ill-defined purple on a dull yellowish-green background; ferric chloride/perchloric acid and *p*-dimethylaminobenzaldehyde gave mauve colours, both of which faded in the centre of the spots on continued heating to give shades of brownish or greenish-yellow: IAN controls gave similar reactions when treated under the same conditions. Pending a more specific

characterization, one may reasonably assume from this evidence that IAN is present in this fraction.

On the histogram there are two other zones of growth promotion. The one at the IAA region was further investigated as follows: the present experiment was repeated and the zone corresponding to R_f 0.29–0.44 removed, eluted

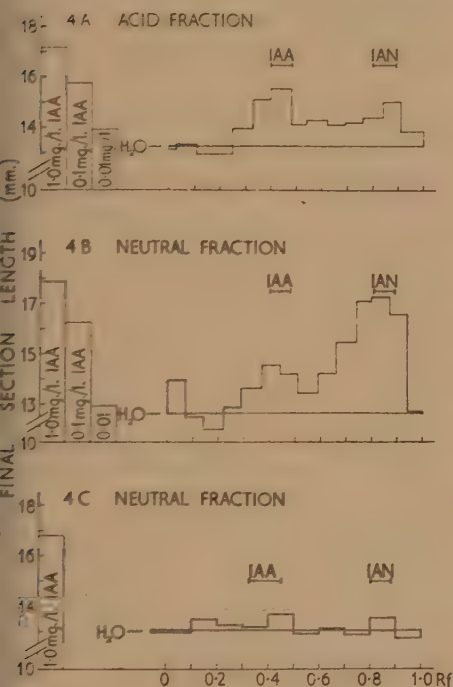


FIG. 4.

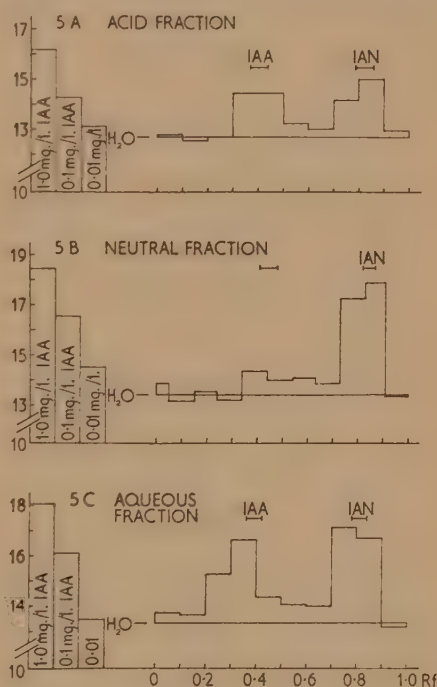


FIG. 5.

FIG. 4. Chromatography in ammoniacal isopropanol of ether extracts (fractions 3.1 and 3.2) of the heat-treated (97°C . for 25 minutes) syrup of fraction 3. 4c:—Chromatography of a water extract of a chromatogram zone corresponding to R_f 0.29–0.44 in 4b.

FIG. 5. Chromatography in ammoniacal isopropanol of ether extracts (fractions 3.3 and 3.4) of the syrup of fraction 3. 5c is the aqueous remainder (fraction 3.5) after ethereal acid and neutral fractions have been removed.

with water, the eluate applied to a fresh paper under a stream of warm air ($30\text{--}40^{\circ}\text{C}$.) and the chromatogram developed in isopropanol/ammonia. The result (Fig. 4c) is essentially identical with that of Fig. 3a in which a growth promoter at the IAN region originates from a precursor in the growth-active zone at the IAA position. It is of interest that a third and possibly a fourth active zone in Fig. 4c have appeared at R_f 0.1–0.2 and R_f 0.6–0.7 respectively, and these cause the histogram to resemble Fig. 3e in pattern; these zones will be considered with other data in the discussion.

In Fig. 4b the third zone of promotion at R_f 0.0–0.07 does not coincide with U.V. fluorescence; also, it has not been possible to detect a reaction at this position with chromogenic sprays. If this histogram is compared with Fig. 2a,

it would appear that a new growth promoter has been formed by heat treatment of the syrup. If this is true, then the promoter in Fig. 1*b* at Rf 0.008 may have been formed in a similar manner: this hypothesis is examined in the following experiments.

The acid fraction (Fig. 4*a*) shows growth promotion at IAA and IAN zones. With ferric chloride/perchloric acid spray, a pink colour was developed at the former and a faint pink at Rf 0.73. This suggests that heat treatment has produced some IAA; however, further investigations are required to confirm its presence.

To examine the formation of the active zone at the starting-line in Fig. 4*b*, it was decided first to repeat the last experiment using non-heat-treated syrup of fraction 3. Approximately 250 mg. syrup were separated into acid and neutral fractions (3.3 and 3.4 respectively) after the manner of fractions 3.1 and 3.2, and half of each fraction chromatographed in *isopropanol/ammonia* (Fig. 5*a* and *b* respectively). The acidified-bicarbonate aqueous remainder from this separation (fraction 3.5) was brought to pH 6.2 with barium hydroxide, centrifuged, concentrated to a thin syrup, and slightly less than half of this concentrate chromatographed (Fig. 5*c*).

Results of chromatography of the neutral fraction (Fig. 5*b*) indicate the presence of IAN, ferric chloride/perchloric acid and nitrous/nitric acid sprays confirming this. Activity at Rf 0.005 is small, and the result is consistent with the heat-treatment hypothesis of the formation of the growth promoter at this position; however, it should be borne in mind that the syrup of fraction 3 has had some heat treatment during its preparation, therefore an examination of fraction 4 would be preferable, although this has had heat treatment too. This examination is reported in the next paragraph. Histograms of fractions 3.3 and 3.5 (Fig. 5*a* and 5*c* respectively) are essentially similar, with growth promotion at the IAA and IAN positions.

On examining the frozen stock solution of fraction 4, it was found that 10 ml. of a water-miscible light-brown 'oil' had separated from it. Bioassay showed that this material (fraction 4.7) was inactive when tested at 285 mg./l. and lower concentrations, but after the oil had been ether-extracted and the extract chromatographed, activity was obtained (reported later). A 50 ml. aliquot of fraction 4, taken after removal of the 10 ml. oil, was acidified to pH 3 with sulphuric acid, extracted with 5 × 50 ml. ether, the ether extract concentrated to 50 ml. and then equally divided into two parts. One portion was shaken with 30 ml. sodium bicarbonate solution, the bicarbonate phase re-extracted with 2 × 30 ml. ether, and the pooled extracts concentrated to a small volume (fraction 4.3): the acid material (fraction 4.2) was also examined; this was obtained in the usual manner except that 2 × 30 ml. followed by a 50 ml. ether extraction of the acidified bicarbonate solution was used. The remaining portion was not shaken with sodium bicarbonate solution, but was concentrated to a small volume (total ether-soluble material, fraction 4.1). All concentrates were chromatographed in *isopropanol/ammonia*.

Results (Fig. 6*a* and *b*) differed from those of the two preceding experiments

(cf. Figs. 4*b* and 5*b*). Extensive zones of inhibition are shown on the histograms, and coleoptile sections in some of these zones showed loss of turgidity, distortion, and discoloration. U.V. fluorescence did not suggest that this inhibition could be caused by a high supra-optimal concentration of IAN; in fact the zone of minimum inhibition in the IAN region suggests that this hormone is partially alleviating the effect of an inhibitor.

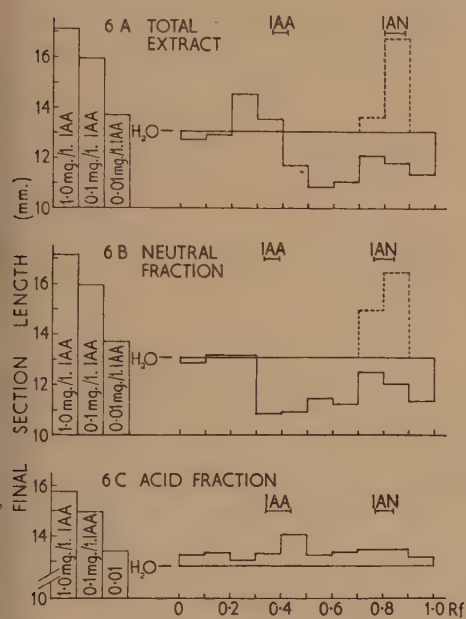


FIG. 6.

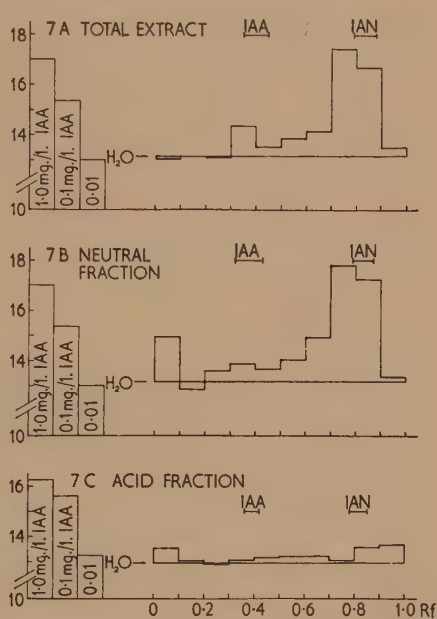


FIG. 7.

FIG. 6. Chromatography in ammoniacal *isopropanol* of ether extracts (fractions 4.1 to 4.3) of the water-soluble fraction 4. The latter differed from the material used in Fig. 5 in that it was not concentrated to a syrup before ether extraction. Responses at $\times 10$ dilution are indicated by broken lines.

FIG. 7. Chromatography in ammoniacal *isopropanol* of ether extracts (fractions 4.4 to 4.6) of the heat treated (99°C . for 25 minutes) fraction 4. The material was similar to that used in Fig. 6 except that the fraction was distilled to a syrup before heat treatment.

Comparison with preceding experiments suggests that distillation of fraction 3 to form a syrup removed a volatile inhibitor: this inhibitor has neutral rather than acidic properties (cf. Fig. 6*b* and 6*c*). In order to test this suggestion, another 50 ml. of fraction 4 were taken and distilled until 34 ml. distillate were collected. Bioassay of this distillate (Fig. 8*d*), using wheat coleoptile sections, clearly shows that an inhibitor was present: it is probable that only part of this substance was condensed and collected during the distillation under reduced pressure; even so, the difference between responses at the highest concentration and the water control is highly significant ($\gg 1$ per cent. probability level). It is seen from Fig. 6*a* and *b* that inhibition did not occur at R_f 0.0-0.2. Both histograms show no activity in this zone, and it would thus

seem that heat treatment of fraction 4 is necessary for growth promotion to be shown at Rf 0.0.2. It may be added that 50 ml. of fraction 4 is quite adequate to show this effect, this volume being equivalent to approximately 1,000 mg. of the syrup of fraction 3.

TABLE

Peaks of growth promotion on histograms of Figs. 1-7 which cannot be ascribed to IAN, IAN-precursor, or accelerator- α

Isopropanol/ammonia						n-butanol/ammonia		
Fig.	Fraction	Rf	Fig. 1a	Fraction Acid	Rf 0.24-0.33	Fig.	Fraction	Rf
2a	Aqueous	0.15-0.23						
3c	"	0.09-0.2	3e	Aqueous	0.58-0.65			
3e	"	0.12-0.25	4a	Acid	0.54-0.62	2b	Aqueous	0.38-0.49
4c	Neutral	0.1-0.2	4c	Neutral	0.6-0.7	3b	"	0.33-0.47
5b	"	0.15-0.24	5b	"	0.54-0.63	3f	"	0.29-0.43
6c	Acid	0.1-0.2	7c	Acid	0.5-0.7	2b	"	0.57-0.67

One may now test the heat-treatment hypothesis mentioned above. A second 50 ml. aliquot of fraction 4 was taken and concentrated to a thin syrup which was heated at 98-100° C. for 25 minutes, cooled, diluted to 30 ml. with water, and the pH reduced from 5 to 3 with sulphuric acid. The remaining steps yielding fractions 4.4-4.6 are similar to those giving fractions 4.1 to 4.3. Results clearly show that growth occurs in the neutral fraction 4.6 (Fig. 7b) at Rf 0.0.1, but the total extract (Fig. 7a), which has had heat but no bicarbonate treatment, has no corresponding promotion. It would thus appear that this growth promoter has been formed in at least two stages, one involving heat and the other bicarbonate treatment.

The acid fractions 4.5 and 4.2 (Fig. 7c and Fig. 6c) have low activity. This has occurred through the introduction of the additional ether extraction and separation of acid substances; the precursor or hormone at the IAA position is a water-soluble substance (see discussion and note at end of paper).

After the completion of this last experiment, no further work was carried out on fraction 4 for a period of eight months: during this interval, it was stored at -15° C., and periodically the light-brown oil which separated from it was removed until the frozen solution was almost colourless.

Since the growth promoter at Rf 0.0.1 of the neutral fraction 4.6 (Fig. 7b) was present also, due to incomplete separation, in the acid fraction 4.5 (Fig. 7c) at low concentration, it was thought that it might be identical with accelerator- α of the acid fractions of Bennet-Clark and Kefford (1953). To test this possibility, it was decided to examine the growth of cress roots in the neutral hormone (fraction 4.6, Rf 0.0.1) to see whether the characteristic α root-growth promotion reported by these workers was obtained.

A 100 ml. aliquot of fraction 4 which had been stored for eight months was subjected to the same procedure that had yielded fractions 4.4-4.6. On

chromatography and bioassay of the neutral fraction corresponding to fraction 4·6, growth promotion at Rf 0·0·1 was only 0·35 mm. above the water control (13·1 mm.) compared with 1·9 mm. in Fig. 7*b*; on the present occasion coleoptile sections were more sensitive to applied IAA, having a response of 17 mm. at 0·1 mg./l. Another experiment repeated with appropriate dilutions

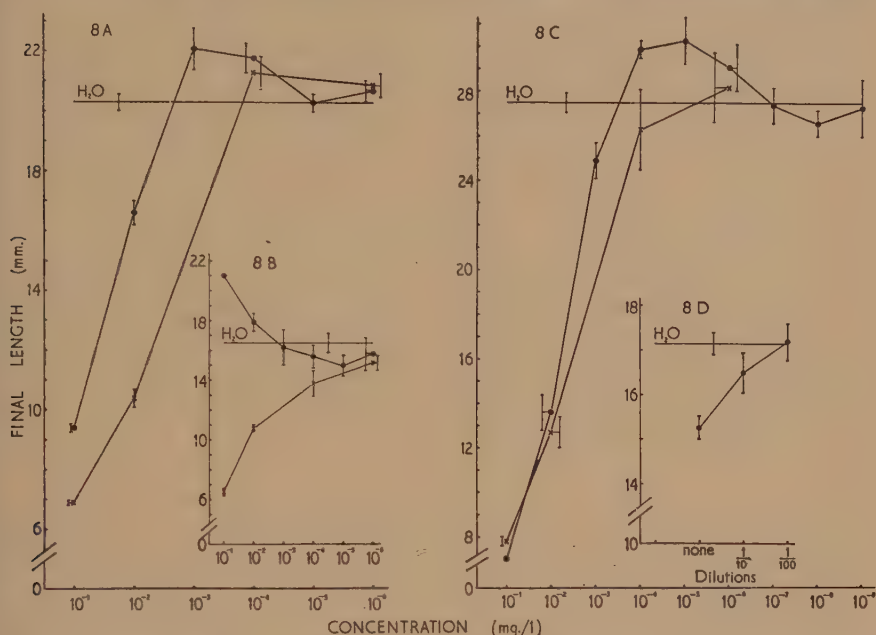


FIG. 8*a-c*. Growth of cress roots on agar containing either *W* (8*a* and *b*, —●—; see Discussion) or IAN-precursor (8*c*, —○—). With the latter, 0·5 ml. of the eluate used for chromatography in Fig. 3*e* and *f* was diluted to 6 ml. and incorporated into 3 per cent. agar. Eluate concentrations are arbitrary and are not related in different figures. A 1·5 per cent. agar (water) control and an IAA activity curve (—×—) are included. Vertical lines indicate the standard error above and below each mean; one point (8*b*, —●— at 10⁻¹ mg./l.) has a zero value. Means were determined by 6 replicates, except for the water control which results from 24.

FIG. 8*d*. Response of wheat coleoptile sections in the distillate resulting from concentration of fraction 4 to a syrup. The water control was determined by 30 section replicates.

of the test solution confirmed this loss of activity. Although activity was low, it was possible to obtain suitable cress-root activity curves (Fig. 8*a* and *b*) for comparison with those of Bennet-Clark and Kefford; these will be considered in the discussion.

Before assuming that the precursor of this neutral hormone had changed during storage, it was decided to examine the oil fraction 4·7 which had separated from fraction 4. 1 ml. oil was diluted to 3 ml. with water, and then treated as in the separation of fractions 4·4 to 4·6. The neutral fraction showed similar composition and activity to those of fraction 4·6 so that it is clear that some of the precursor giving rise to this neutral hormone had separated from the frozen aqueous fraction in the oil fraction 4·7.

To confirm the results of the last experiment, it was repeated, but with the

following modifications. Two separate 1 ml. aliquots of oil were taken and immediately after heat treatment cold solutions of normal sodium hydroxide or normal sodium bicarbonate were added. Response at Rf 0.0-0.1 in the bicarbonate-treated neutral fraction was very similar to that in the preceding experiment, but sodium hydroxide caused reduced activity which was confirmed by appropriate dilutions. Thus, it would appear that more favourable conditions for hydrolysis do not result in an increased hormone yield.

In this experiment it was noted that the NaOH-treatment chromatogram, when examined in U.V. light, had a more intense fluorescence at the position of IAN. Since the latter possibly indicated an increased yield of precursor-liberated IAN, it was decided to compare the biological activities of this part of the bicarbonate and hydroxide-treatment chromatograms. Using the histogram of the last experiment as a guide, IAN segments (Rf 0.6-1.0) from each chromatogram were eluted under identical conditions, and then assayed using dilutions to obtain the whole activity curve. The results, when plotted graphically, indicated that sodium hydroxide treatment had increased IAN liberation by a factor of $\times 2.2$ compared with sodium bicarbonate treatment. Thus, it would appear that more favourable conditions for hydrolysis increase the IAN yield.

DISCUSSION

Hormones and precursors in the aqueous fraction. Examination of the water-soluble, ether-insoluble portion of a cabbage extract has shown that this fraction has auxin activity. Chromatography has resulted in two zones of growth promotion (*A* and *B*), *B* having the same Rf as IAN. The hormone in this zone is ether-soluble, neutral, and has the Rf value, U.V. fluorescence, and chromogenic reactions of IAN with which it is believed to be identical.

The presence of this hormone in the aqueous fraction suggests that it has been produced from a water-soluble precursor: evidence for this is shown in Fig. 3. Rechromatography of the material of zone *A* results in the appearance of *B*, suggesting that it has originated from a precursor at *A*. Ammoniacal *n*-butanol gives better separation of *A* and *B*, and therefore Fig. 3*c-f* gives a clearer demonstration of production of the nitrile from the precursor.

Before considering this precursor system in greater detail, it is desirable for subsequent discussion first to indicate how IAN may have been freed from the precursor during experimentation. One method of IAN liberation appears to be by alkaline hydrolysis. For example, it was noted that when separation of acid and neutral fractions was effected with sodium hydroxide rather than bicarbonate, the IAN yield was increased. A second example, showing liberation under the influence of ammoniacal chromatography, is illustrated by Fig. 3*c-f*, although it is possible that some of this IAN could have been liberated during elution of the primary chromatogram (see below).

Another method of nitrile liberation is by heat treatment of its precursor. Preliminary experiments showed that heat treatment (97-100° C. for 25 minutes) of the aqueous fraction 4 at pH 5.6 increased the amount of IAN

on a chromatogram developed in *n*-butanol/ammonia, but conversion to nitrile was only partial and the precursor could still be readily demonstrated by bioassay; thus, under these conditions the IAN-precursor is not completely unstable.

It was noticed during the preparation of fraction 4 that there appeared to be a slow liberation of IAN at room temperature (15° C.). Each time the ether-extracted aqueous fraction was re-extracted with more ether, IAN was detected in the ether extract. It may be that IAN is being liberated by a hydrolysis which can take place to a limited extent under these mild conditions, but this reaction is not accelerated to completion by heat at near-neutral pH values.

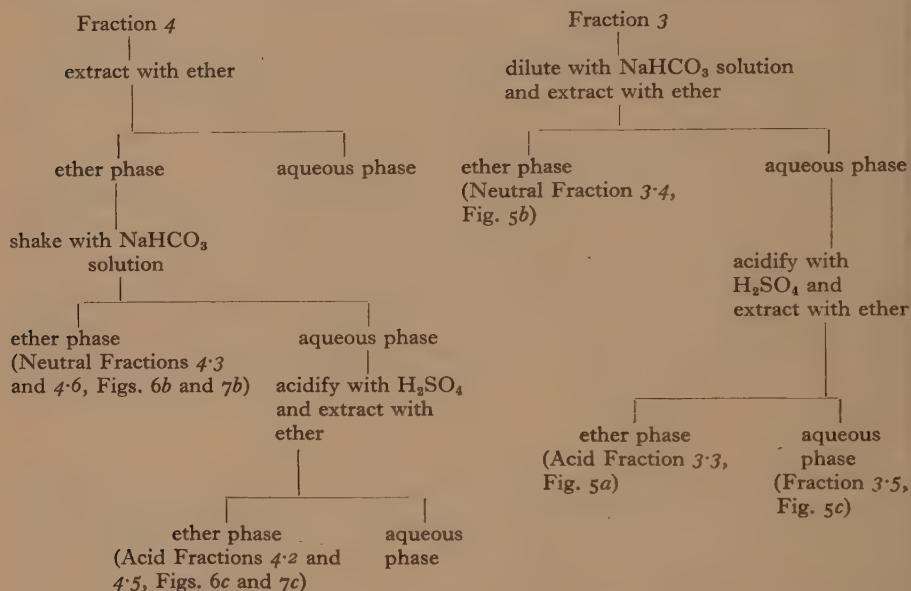
Returning to the chromatography of the IAN-precursor, if IAN at *B* is being liberated from a precursor at *A*, it is pertinent to query the nature of the growth promoter in this latter zone. One may suggest that the precursor ran as such on the chromatogram, but that conversion to IAN occurred on drying; however, ferric chloride/perchloric acid and nitrous/nitric acid sprays clearly indicate that free IAN was not present on this part of the chromatogram. Other alternatives are that the precursor itself is active, or that it is inactive but is converted to IAN either within the coleoptile cells, or in the solution during elution and assay.

In view of the instability of the precursor at room temperature, one must attribute some growth to IAN freed during elution and assay, but whether all growth can be thus accounted for is uncertain. If one accepts the hypothesis that IAN is only active in growth by virtue of its conversion to IAA within the cell (Thimann, 1953), then it is quite possible that the IAN-precursor is also converted to IAN intracellularly, and that this is a further step of auxin biogenesis. Although this explanation would account fully for growth at *A* on the chromatograms, there may be other contributory factors. It has already been noted in Figs. 4*c* and 3*e* that rechromatography of zone *A* yields other growth promoters besides precursor-liberated IAN. While these may be associated with the IAN-precursor, it is also possible that they indicate the presence of other active substances at *A*; thus, it is possible that growth at this position results from the action of more than one substance.

Acid and neutral components of the water-soluble fraction. During separation of the acid and neutral fractions 3.3 and 3.4, the precursor may have appeared in both. Comparison of the histograms in Fig. 5 suggests that it has mainly entered the acid fraction assuming that activity at the IAA position is caused by the precursor. If this is correct, then rechromatography of the IAA zone should result in stimulation at this same position, and also at IAN due to chromatographic liberation of this hormone. Although this experiment has not been carried out on the acid fraction, Fig. 4*c* shows that it is true for the heat-treated neutral fraction; the heat treatment does not influence the validity of this conclusion.

Since the separation technique permitted the water-soluble precursor to enter the neutral ethereal fractions of Figs. 4-7, then it will be present also in

the corresponding acid fractions: this may be shown by comparing Figs. 6*c* and 7*c* with Fig. 5*a*. The experiments of these figures differ in their methods of separation of acid and neutral fractions; these are shown below. It is seen that fraction 4 was extracted with ether, and this ethereal extract then separated into acid and neutral materials with sodium bicarbonate solution, whereas fraction 3 was directly separated into acid and neutral materials



without an initial ether extraction. The effect of this extra step on a substance poorly soluble in ether will be to reduce its concentration in the acid and neutral fractions of Figs. 6 and 7 compared with those of Fig. 5. One may therefore expect histogram patterns in the acid fractions to be similar, and if peaks in Fig. 5*a* result directly or indirectly from water-soluble substances, section response in Figs. 6*c* and 7*c* will be relatively smaller than in Fig. 5*a*. It may be added that quantitative differences in the amount of extract used to obtain these acid fraction chromatograms tend to enhance the significance of the results rather than diminish it (see note at end of paper).

It has been seen that growth promotion at *Rf* 0-0.1 on neutral fraction histograms (Figs. 1*b*, 4*b*, and 7*b*) only occurs after the syrup has been heated and treated with sodium bicarbonate solution. A simple hypothesis to account for this observation is shown below. One may suppose that a substance *U* in



the water-soluble, ether-insoluble fraction is changed by heat to yield another compound *V*. *V* reacts in the presence of sodium bicarbonate solution to yield *W* which causes or leads to cell elongation. *V* may be active or inactive;

however, if it is active then the histogram of Fig. 7*a* suggests that its Rf must be the same as that of the IAN-precursor or IAN.

The conditions under which the second reaction forming *W* take place are very mild, and this suggests that *V* is quite a reactive substance. It is evident from Fig. 7*a* that chromatography with ammonia does not lead to the formation of *W*, and it is possible that this reaction is not a hydrolysis, but a more specific reaction with sodium bicarbonate. This conclusion is supported by the experiment in which sodium hydroxide was used instead of bicarbonate to separate acid and neutral fractions from fraction 4·7 where the yield of *W* was reduced (see p. 232). Figs. 4*b* and 7*b* indicate that *W* has entered the neutral fraction, and that little or none is in the acid fraction. This suggests that *W* is ether-soluble rather than water-soluble, for had *W* been water-soluble one would have expected an appreciable quantity to have entered the acid fraction. Thus *W* is a neutral ether-soluble substance, but its Rf value suggests that polar groups are present in the molecule.

Growth regulators in the ethereal fraction. Chromatography of fractions 1 and 2 has revealed patterns of growth promotion which differ from those obtained by certain other workers (see Kefford, 1955).

Bennet-Clark and Kefford (1953), examining the acid fraction of a range of plant material, reported growth promotion at Rf 0·0–0·2 on chromatograms developed in ammoniacal isopropanol; it was assumed to result from a single substance termed accelerator- α . Other workers have subsequently reported its presence in various plant materials, but at Manchester it is not normally detected. The work of Bennet-Clark and Kefford has been kindly made available to the Manchester workers in a detailed form (Kefford, 1954), and certain critical experiments not reported in the press have enabled a new interpretation of α to be made.

Stimulation due to α varied considerably in the plant extracts examined by these workers. Their methods of extraction and separation with sodium bicarbonate have been described (Kefford, 1955), but it should be mentioned that the technique did not prevent neutral substances from appearing in the acid fraction. Kefford (1954) carried out an experiment in which 100 μ g. IAN in ether were extracted with bicarbonate solution, and bioassay showed that some IAN had entered the bicarbonate.

At one stage of their work an attempt was made to obtain an acidic extract with fewer impurities by the following modification of their technique. 50, 100, and 125 g. etiolated pea shoots were extracted in the routine manner until extracts in 5 per cent. sodium bicarbonate solution were obtained. These were acidified to pH 7 and extracted with 3×15 ml. ether before being further acidified to pH 3 and again ether-extracted. The 50 and 100 g. extracts taken at pH 7, and the 125 g. extract at pH 3 were chromatographed and bioassayed. The 50 g. extract gave marked stimulation at α , while the 100 g. extract gave growth which was slightly less than a sucrose control. The 125 g. extract was inactive in the region of α .

These results were interpreted with the aid of their accelerator- α activity

curve (Bennet-Clark and Kefford, loc. cit., Fig. 2a). It was suggested (Kefford, 1954) that the 100 g. fraction at pH 7 showed inhibition due to high hormone concentration, whereas this was absent with the 50 g. fraction. α is considered to be an acid, although its marked entry into a fraction drawn at pH 7 is thought to indicate acidic properties that are weaker than those of IAA.

Although this interpretation by these workers is appropriate in view of the limited data available to them, it is felt that the present work enables a new interpretation of α to be made. It is suggested that this hormone is not an acid, but the neutral growth substance W at Rf 0.0-0.1 in Figs. 1b, 4b, and 7b. The reason for its occurrence in Bennet-Clark and Kefford's acid fractions lay in their technique of separation with bicarbonate; this is clearly shown by Kefford's analogous experiment with IAN.

With regard to their pea shoot experiment, the 50 g. extraction gave 82 per cent. elongation at α (control sucrose + 0.125 mg./l. IAA 72 per cent.; sucrose alone 30 per cent.). This shows that their α behaves essentially as a neutral substance. With the 100 g. extraction, inhibition due to high hormone concentration may be the correct interpretation; alternatively, no α may have been present owing to insufficient heat treatment before bicarbonate treatment. Results of the 125 g. extract drawn at pH 3 show that section response is identical with the sucrose control. This agrees with expectation, for after α has been twice separated at pH 8 and 7 one would expect little or none in the acid fraction. Alternatively, of course, no α may have been formed.

Thus, it is seen that results of Bennet-Clark and Kefford are consistent with this interpretation of α . A method of testing whether this is correct would be to compare the effect of α and W on root growth. If W promotes growth in the same manner as α , then this could be cited as evidence supporting this interpretation. Fig. 8a clearly shows that cress root growth in W is essentially identical with the pea root growth in α obtained by Bennet-Clark and Kefford (loc. cit., Fig. 2a). A greater stimulation above water was obtained in a second experiment (Fig. 8b), but the concentration of W was lower and was too weak to give hormone-induced inhibition: it must be emphasized that the concentrations of W in these two figures are arbitrary and unrelated, and have only been plotted in this manner for convenience.

It is interesting that a similarly shaped activity curve (Fig. 8c) is given by the eluate taken from the IAN-precursor portion of a chromatogram developed with *n*-butanol/ammonia. This is strong evidence that the activity in the IAN-precursor zone bears some relationship to α . However, Fig. 2a shows that the syrup used for the butanol chromatogram contained no α , so that if there is some relationship it must be with the precursors of α , e.g. substance U , which is water-soluble. α is reported to be present in the roots of maize seedlings (Kefford, 1954 and 1955), which from unpublished work in this laboratory do not appear to have an IAN system. It follows, therefore, that cabbage must have two hormone systems, one related to the nitrile, the other to α .

Having considered α in some detail, the remaining acid and neutral hormones will be examined. It has already been noted that little or no free IAA

appears to be present in the acid fraction (Fig. 40). Before concluding that this reflects the true state in the plant, it is necessary to consider whether leaching during extraction has occurred. It will be recalled that extraction with plants is carried out approximately pH 3 for 48 hours, the chance of pH being influenced by the report that acidic precursors are present in certain plant materials (Borla, 1953). However, Hahn and Minard (1954) have examined the stability of IAA in phosphate buffers pH 1.5-9, after 1, 3, and 76 hours incubation, and found that after 76 hours at pH 3 approximately only 90 per cent of the applied IAA can be recovered; their data indicate that over short periods at low pH values IAA is stable, but over long periods considerable leaching occurs. Therefore, it is clear that even if a large amount of free IAA was present in the 100 g cabbage leaves used in this investigation, it is probable that one would not detect it with the present extraction technique.

It has been noted that the only evidence of IAA in this plant material was obtained by leucine color or perchloric acid spray of the chromatographed hexane-treated aqueous syrup (Fig. 40). However, treatment at 100°C. is not mild and in the absence of other supporting data, care should be taken before concluding that the IAA formation is not an artifact. Before IAA production from the aqueous fraction can be accepted as fact, further investigations are required.

Fig. 40 shows growth promotion at Rf 0.95-0.99, with tailing in Rf 0.24. The peak lies close to IAA and partially overlaps it. It seems likely that this peak is due to IAA, but there is some uncertainty regarding the mode of its entry into the acid fraction. The evidence suggests that it has not been liberated from its acidic precursor, but that free IAA entered during separation with sodium bisulfonate solution.

It is possible that the tail of activity behind the Rf of IAA is not connected with IAA or its precursor but with other growth promoters which appear to be present in the cabbage in small amounts. Several histograms have small peaks which cannot be accounted for by growth promoters already discussed. While many of these peaks are too small to merit individual consideration on histograms, when examined collectively (see Table) they acquire a greater significance. It is not possible to state with certainty how many additional growth substances are present, but there would appear to be at least two, and there are probably more.

The authors wish to thank the Agricultural Research Council for grants which have enabled this work to be carried out, and Professor A. C. Harland, F.R.S., for generous facilities in the Department of Botany, and Mrs. V. Shaw for technical assistance with the experimental assays, manuscript, and figures.

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Note added in proof

One of us (J. A. B.) suggests an alternative explanation, that the ether-soluble activity obtained after treatment with bicarbonate (Figs. 4a, 4b, 5a, 5b, 5c) is due to slight breakdown of the water-soluble compounds in alkali before extracting with ether, and not to partitioning of the original compounds into ether. The increased activity is particularly noticeable in the acid fractions (Figs. 4a and 5a compared with Figs. 6c and 7c). The activity in Figs. 6a and 7a is probably due to slow breakdown of the water-soluble compounds with time and temperature during manipulation. The instability of these compounds has been demonstrated elsewhere in the paper.

Studies in Plant Growth Hormones

V. CHROMATOGRAPHY OF HORMONES IN EXCISED AND INTACT ROOTS OF TOMATO SEEDLINGS

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Received 21 October 1955

SUMMARY

1. An examination has been made of the hormones present in extracts of excised roots and intact seedling roots of tomato. Acid and neutral ether-soluble fractions and the ether-insoluble aqueous fraction were chromatographed, and the chromatograms assayed using oat coleoptile sections.

2. The pattern of hormone activity in excised roots differed little from that in seedling roots.

3. On chromatograms of the aqueous fraction developed in *isopropanol*/ammonia, growth promotion occurred at the position of 3-indolylacetic acid (IAA, Rf 0.5) and sometimes at the position of 3-indolylacetonitrile (IAN, Rf 0.8). When the IAA zone was eluted off the paper and rechromatographed, it formed the IAN zone and another zone of promotion at Rf 0.1-0.2.

4. When the aqueous fraction was developed in *n*-butanol/ammonia, promotion occurred at the position of IAA (Rf 0.15), at Rf 0.5, and at the position of IAN (Rf 0.85). These zones have been called X, Y, and Z respectively. They were also formed when the IAA zone in *isopropanol*/ammonia was rechromatographed in ammoniacal *n*-butanol. It is shown that X and Y are interconvertible, and that each can form Z on rechromatography; also, there is some evidence that Z can form X and Y. When Z was separated into ether-soluble (acid and neutral) and ether-insoluble fractions with sodium bicarbonate solution and chromatographed in *isopropanol*/ammonia, growth resulted at the position of Z in the neutral and aqueous fractions, but in the acid fraction it occurred at Rf 0.24-0.35. Comparison with other chromatograms indicates that this last zone does not occur in the aqueous fraction but has been formed as a result of extraction with sodium bicarbonate solution.

5. The zones found in the aqueous fraction also occurred in small quantities in acid and neutral ethereal fractions in a number of experiments.

6. The ethereal fractions gave no chromogenic reactions with ferric chloride/perchloric acid, nitrous/nitric acid, or *p*-dimethylaminobenzaldehyde (MeAB). In the aqueous fraction only MeAB gave a reaction (yellow) which showed any consistent correlation with biological activity. A yellow colour with this reagent is not a characteristic chromogenic reaction of indole compounds. It is suggested that a non-indole hormone system may be operating in tomato roots.

INTRODUCTION

EARLY studies on the production of auxin in roots led to considerable controversy; Cholodny (1924), Boysen-Jensen (1933*a* and *b*), Nagao (1936), and

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others were of the opinion that there is production of growth substances in root tips, while Bünning (1928), Gorter (1932), Went (1932), and Thimann (1934) opposed this view. This controversy extended to excised roots cultured in sterile media. Fiedler (1936) reported that no hormone could be detected in maize and pea during active growth, while Nagao (1937 and 1938), working with the same material and with sunflower, reported its continuous production. Other studies on pea (van Overbeek and Bonner, 1938; van Overbeek, 1939*a* and *b*) supported the observations of Nagao.

Over the following decade little biochemical work was carried out on roots; however, the presence of a hormone system was accepted, and it was generally assumed that 3-indolylacetic acid (IAA) was the sole or dominant auxin in it. This assumption has been queried by Audus and Shipton (1952) who studied the growth of pea root sections in IAA and other substances. The results of these workers could not be interpreted in terms of current hypotheses on auxin/root growth relationships, and this led them to question whether IAA is present in roots and whether other substances may not be the natural auxins.

By the application of paper chromatography to the separation of growth substances in plant extracts, Bennet-Clark and Kefford (1953) were able to show that other acidic regulators besides IAA are present in root extracts of pea, maize, and broad bean: similar results were subsequently obtained by Lexander (1953) using wheat. These substances are described in the preceding paper of this series (Housley and Bentley, 1956).

In the past, excised root culture has not been widely applied to problems of hormone biogenesis, or to problems arising out of the interaction of hormones and other variables in growth. However, Street (1954*a*) has shown that under certain conditions 'physiological ageing' occurs in root cultures of tomato, and that this process probably depends upon change in hormonal balance. Since IAA did not seem to be one of the hormones involved, it was thought that an examination of the natural auxin system would be of interest for these studies.

This paper reports the results of an investigation of growth regulators present in excised tomato roots after several days growth in a standard culture medium. In order that a comparison may be made with the system present in intact roots, the corresponding investigation is also reported for seedlings. Ethereal and aqueous fractions of extracts have been examined by paper chromatography and *Avena* straight-growth bioassay.

MATERIALS AND METHODS

Materials and methods of chromatography and bioassay were similar to those used in the preceding paper (Housley and Bentley, 1956). The method of plant extraction was basically similar; differences in technique are noted in the text.

Excised roots of tomato (*Lycopersicum esculentum* var. Sutton's Best of All) were obtained by growth of 10 mm. clonal tips, derived from clonal sector

cultures, in a modified White's medium for 10–12 days at 27° C. The method of culture has been described by Street (1954b).

Seedlings roots were grown in sand in a greenhouse, watered daily with the culture medium (less sucrose, vitamins, and $\text{Fe}_2(\text{SO}_4)_3$), and harvested after 4–5 weeks.

RESULTS

Chromatography of the water-soluble fraction. 130 g. fresh weight of excised roots were extracted at pH 3 by the usual method except that ether replaced alcohol for extraction. The ether-insoluble water-soluble fraction was concentrated to a syrup and used in the following experiments. The fresh weight in grams of root material from which each extract was derived is shown on the histograms. Owing to the viscosity of the syrup the primary chromatograms were poorly developed and had to be eluted with water, the eluates concentrated, and then rechromatographed before a satisfactory separation could be obtained. To enable a comparison to be made with excised material, an extract of 143 g. seedling roots was also prepared and chromatographed in a similar manner.

Chromatography of the excised root fraction in *isopropanol/ammonia* (Fig. 1a) resulted in optimum stimulation (Rf 0.36–0.48) at the position of IAA, while activity extending to Rf 1.0 suggests that other active substances were present. When sprayed with nitrous/nitric acid, a pale yellow area at Rf 0.36–0.54 appeared; no reactions with ferric chloride/perchloric acid or *p*-dimethylaminobenzaldehyde (MeAB) were obtained.

In order to examine the zone having the same Rf as IAA in greater detail the experiment was repeated using the same loading, and the zone corresponding to Rf 0.36–0.64 in Fig. 1a removed, eluted with water, and half the eluate chromatographed in the same solvent for bioassay (Fig. 1b). It is seen that continuous activity is present with peaks of promotion at Rf 0.11–0.22, 0.34–0.45, and 0.68–0.78. When sprayed with chromogenic reagents no colour was obtained, but bromcresol green gave an acidic reaction at Rf 0.3–0.53. It would thus appear that material at the position of IAA can readily yield active zones travelling at different positions on the chromatogram.

Since experience had shown that chromatography in *n*-butanol/ammonia usually effects a better separation of growth substances in the aqueous fraction, chromatography was next carried out in this solvent. Three active zones (Fig. 1e; X, Y, and Z) were obtained. With MeAB a pink colour was obtained at Rf 0.05–0.1 and pale yellow at Rf 0.1–0.2, while bromcresol green gave an acidic reaction at Rf 0–0.15. Other experiments with this solvent system yielded similar results except that zones X and Y were not always well separated. This suggested that other active substances may be present but were being partially masked by X and Y. An experiment in which chromatography was carried out for 45½ hours so that the solvent front passed off the paper is shown in Fig. 1f. One can roughly deduce the distance travelled by the solvent from the position of the IAA marker so the zone at 31 cm. appears to have

an R_f value of 0.38. This contrasts with the value of 0.45 for Y in Fig. 1e; however, it is not unreasonable to assume that this zone is Y , and if this is

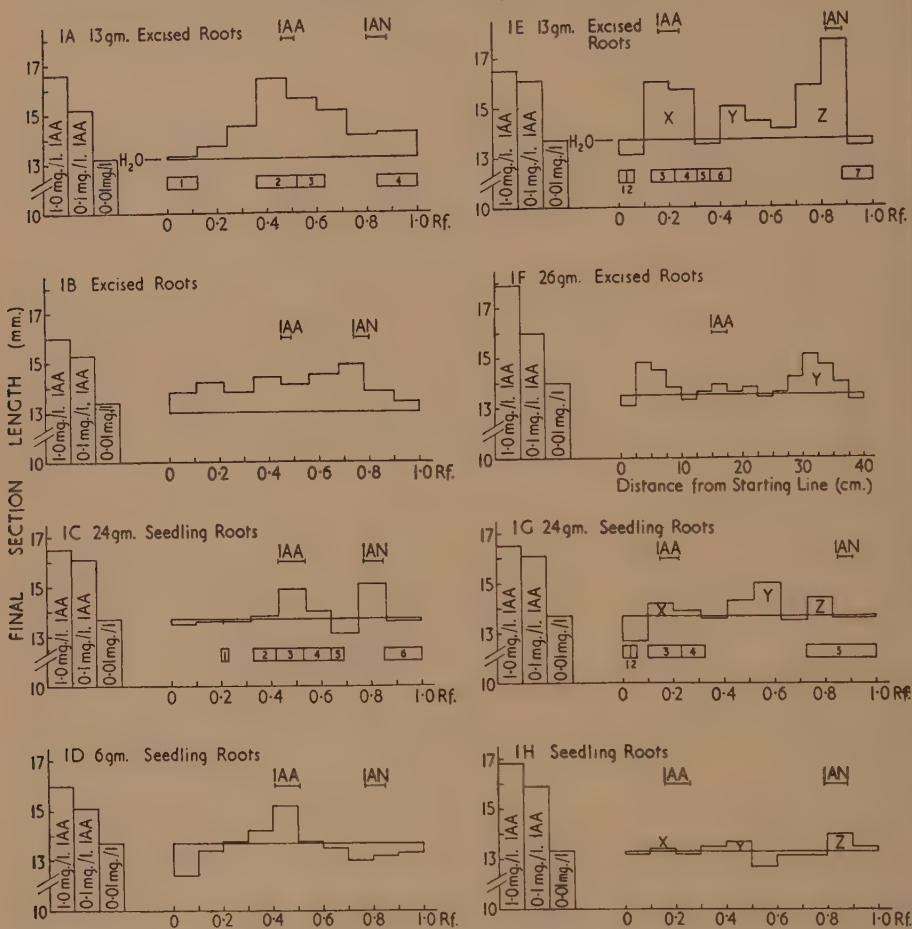


FIG. 1. Chromatography in ammoniacal isopropanol (1a-d) and *n*-butanol (1e-h) of the aqueous fraction of extracts of excised and intact seedling tomato roots. 1b:—the zone in 1a corresponding to R_f 0.32–0.76 was eluted with water, the eluate distilled to 2 ml., and then rechromatographed. 1f:—chromatography was carried out for 45½ hours; the solvent front passed off the paper. 1h:—the zone in 1d corresponding to R_f 0.3–0.5 was eluted with water and the eluate rechromatographed. U.V.—fluorescence on some of the chromatograms is shown below. 1a:—zone 1, pale brown pigment; 2, pale blue with purple; 3, light blue; 4, pale blue. 1c:—zone 1, light blue; 2, pale blue; 3, pale purple; 4, pale blue; 5, pale pinkish-blue; 6, pale blue. 1e:—zone 1, pale green; 2, light blue; 3, pale blue; 4, deep purple; 5, pale blue; 6, pink; 7, pale blue. 1g:—zone 1, pale green; 2, light blue; 3, pale blue with purple; 4 and 5, pale blue.

true, then it is clear from Fig. 1f that there are at least three other growth promoters between Y and the starting-line, one of which has the same R_f as IAA. It may seem peculiar that the largest peak of activity (2.5–7.5 cm.) does not have the R_f of IAA (cf. Fig. 1e); however, it is shown later in this section

that *X* and *Y* are probably interconvertible, and it is quite possible that all these zones are interconvertible and that small differences in technique have caused these growth substances to be present in these particular proportions.

Chromatography of seedling root extracts in *isopropanol/ammonia* (Fig. 1*c* and *d*) and *n*-butanol/ammonia (Fig. 1*g*) gave results which broadly resemble those in Figs. 1*a* and *b*, and 1*e* respectively. With ammoniacal *isopropanol*, it is clear that the zone in Fig. 1*b* at Rf 0.11–0.22 is missing, but the two remaining zones are present in Fig. 1*c* and only one in Fig. 1*d*. Results with chromogenic sprays in Fig. 1*g* were similar to those in Fig. 1*e*, while in Fig. 1*c* MeAB gave yellow and pale blue colours at Rf 0.42–0.48 and 0.74–0.83 respectively, and bromocresol green an acidic reaction at Rf 0.0–0.08 and 0.4–0.55.

Although there appears to be present on the chromatograms an unstable system of inter-convertible growth promoters which may cause considerable variation in growth promotion pattern, the hormone system of excised roots does not seem to differ markedly from that of intact seedling roots.

Since chromatography in *isopropanol/ammonia*, and rechromatography of the IAA zone in this solvent (Fig. 1*b*), may give rise to a number of peaks of growth promotion, it was decided to examine this zone when rechromatographed in *n*-butanol/ammonia. A large chromatogram containing seedling root extract was developed in ammoniacal *isopropanol*; one quarter of the chromatogram was bioassayed to locate activity (Fig. 1*d*), and the zone from the remainder corresponding to Rf 0.3–0.5 in Fig. 1*d* was removed, eluted in a minimum quantity of water, and rechromatographed in *n*-butanol/ammonia (Fig. 1*h*). Although activity is low, it is clear from Fig. 1*h* that the pattern of growth promotion is essentially identical with that in Fig. 1*e* and *g*.

In order to examine these zones in greater detail, it was decided to rechromatograph zones *X*, *Y*, and *Z* and determine their pattern of growth activity. Two chromatogram papers were equally loaded with excised root extract and developed in an identical manner in *n*-butanol/ammonia in the same tank. One was immediately bioassayed (Fig. 2*a*), while the other was divided in a similar manner, each segment frozen with a little water, and then stored until the result of Fig. 2*a* was known. Zones corresponding to *X* (Rf 0.09–0.19) and *Y* (Rf 0.38–0.57) were rechromatographed in the same solvent system and bioassayed (Fig. 2*b* and *c* respectively). It is clear from these two figures that the pattern of growth promotion of Fig. 2*a* has been repeated. While it is possible that *X* and *Y* were not completely separated on initial chromatography and that their positions on the second chromatogram were not quite identical with those of Fig. 2*a*, a comparison of the *X* and *Y* zones in Fig. 2*a*–*c* suggests that mutual contamination can scarcely account for the relative magnitude of the responses in these zones; it would seem more probable that *X* and *Y* are interconvertible. However, it may be safely concluded from Fig. 2*a*–*c* that a growth-active substance at *Z* has been changed or liberated from material at *X* and *Y*.

Since *Z* has an Rf similar to that of IAN and the system bears a close analogy to the behaviour of the IAN-precursor system described by Housley

and Bentley (1956) under similar conditions, it was decided to examine the behaviour of *Z* when separation into acid and neutral components is effected. The portion of the second chromatogram corresponding to *Z* (Fig. 2*a*, *Rf* 0.67–0.76) was eluted with 5 per cent. sodium bicarbonate solution, and then thoroughly extracted with ether to give the neutral fraction. The pooled ether extracts were washed with water and the aqueous washings added back

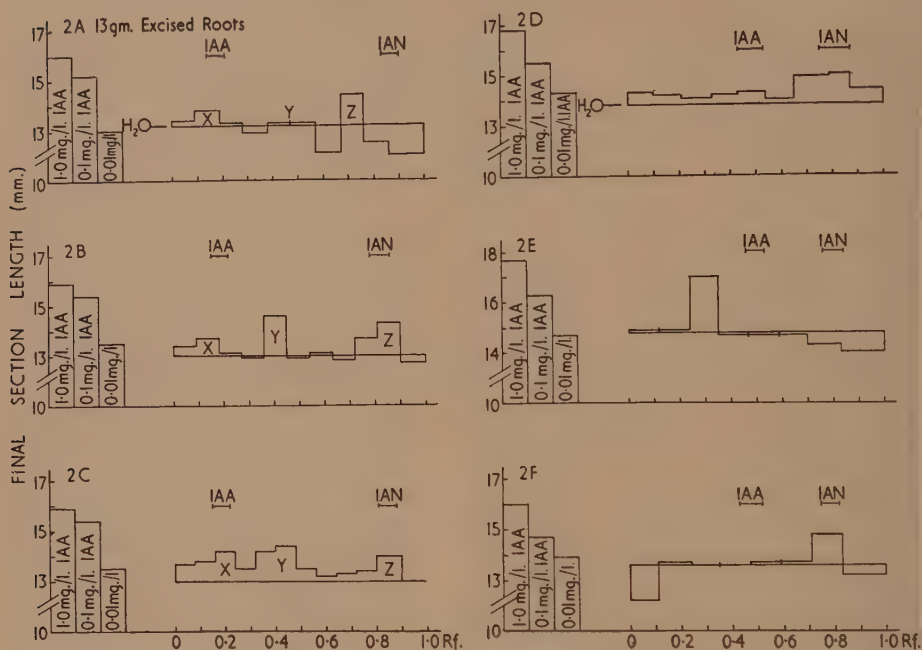


FIG. 2. Chromatography in ammoniacal *n*-butanol (2*a*–*c*) and isopropanol (2*d*–*f*) of the aqueous fraction of extracts of excised tomato roots. 2*b* and *c*:—zones *X* and *Y* (2*a*) respectively when eluted off a chromatogram with water and then rechromatographed. 2*d*–*f*:—neutral, acid, and aqueous fractions obtained when zone *Z* (2*a*) is eluted off a chromatogram with sodium bicarbonate solution and fractionated into ether-soluble, neutral (2*d*), and acid (2*e*) components, and into the aqueous remainder (2*f*).

to the bicarbonate solution. The ether extract was concentrated to a small volume and loaded equally on three spots on a chromatogram which was developed in isopropanol/ammonia. Two spots were used for bioassay (Fig. 2*d*) while the third was sprayed with nitrous/nitric acid; a control IAN spot was also sprayed for comparison. During subsequent heat treatment for chromogenic development, no colour was obtained with *Z* at any stage. From Fig. 2*d*, it is seen that some *Z* has entered the neutral fraction, but in addition there are two other small peaks of growth promotion.

To obtain the acid fraction, the bicarbonate solution was acidified with 5 per cent. sulphuric acid to pH 3 and then extracted with ether; the ether phase was not washed with water before chromatography (Fig. 2*e*). From this figure it is clear that bicarbonate treatment of *Z* has resulted in the formation

of another growth substance. In order to complete the data, the pH of the acidified bicarbonate phase was raised to 5 with barium hydroxide, the precipitated barium sulphate filtered off, the aqueous solution reduced to a syrup and then chromatographed (Fig. 2*f*). It is seen that some Z is still present, but the remainder of the histogram does not show other peaks as in Fig. 2*d*. These results will be further considered in the Discussion.

Chromatography of the ether-soluble fraction. Acid and neutral fractions were prepared from ether extracts of excised and seedling roots. Some of the ether solutions were not washed with water before concentration for chromatography (Fig. 3*b-d, g, and h*) and may therefore contain water-soluble contaminants; others were prepared in the usual manner. The quantity of extract used on each chromatogram is expressed in the same manner as in the preceding section.

Chromatography of the acid fractions in *isopropanol/ammonia* resulted in considerable variation in histogram pattern; selected examples to illustrate the range are shown in Fig. 3*a, b, and d*. Although stimulation in Fig. 3*a* at Rf 0.14-0.3 and 0.86-1.0 is small, responses are significant at the 1 per cent. probability level. In Fig. 3*b* the growth pattern is complicated by the amount of inhibitory material present on the chromatogram; however, there is evidence of a growth substance at Rf 0.1-0.2 in addition to the two clear-cut peaks at Rf 0.3-0.4 and 0.7-0.9. These three peaks are also shown in Fig. 3*d* besides a fourth peak at Rf 0.5-0.6: chromatography in *n*-butanol/ammonia also gave a four-peak histogram (Fig. 3*c*).

Chromatography of the neutral fractions in *isopropanol/ammonia* also resulted in considerable variation in histogram pattern. The examples shown (Fig. 3*e-h*) were selected for the purpose of subsequent discussion; however, the remaining experiments had growth promotion at similar positions except that there were fewer peaks on the histograms. As chromatograms of neutral material were divided for assay according to the pattern of fluorescence in U.V. light, results are less clear than those for chromatograms of the acid material.

On the histograms between Rf 0.6 and 1.0 there appears to be at least one and possibly two peaks of growth promotion (Fig. 3*e and h*), while over the remaining portion there are probably three. Possibly the peaks at Rf 0.27-0.39 (Fig. 3*e*), 0.36-0.48 (Fig. 3*g*), and 0.31-0.42 (Fig. 3*h*) result from the same substance, although it should be noted that the IAA and IAN control spots in Fig. 3*e* have exceptional Rf values in this experiment. From Rf 0.27 in Fig. 3*e* there is tailing activity to the starting line, while in Fig. 3*g* there is activity at Rf 0.11-0.24, a region which approximates closely to a zone where activity has already been noted (Rf 0.1-0.2 in Fig. 1*b, 3b, and 3d*). Finally, in Fig. 3*f and h* there is activity between Rf 0 and 0.1, again a zone where activity has already been noted (see Housley and Bentley, 1956).

Many of the chromatograms in both the acid and neutral fractions were sprayed with ferric chloride/perchloric acid, nitrous/nitric acid, and MeAB; however, chromogenic reactions were never obtained.

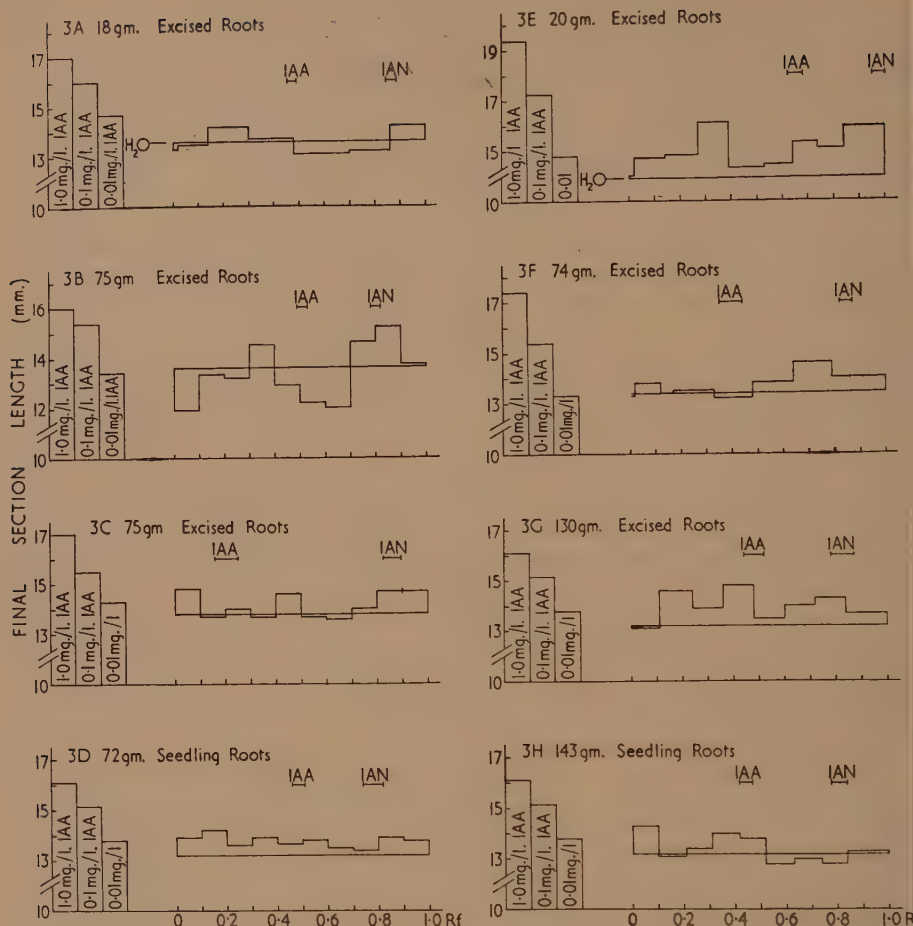


FIG. 3. Chromatography in ammoniacal *n*-butanol (3c) and isopropanol (the remaining figures) of the acid (3a-d) and neutral (3e-h) fractions of extracts of excised and intact seedling tomato roots.

DISCUSSION

Hormones in the aqueous fraction. Examination of the water-soluble, ether-insoluble portion of tomato root extracts has shown that this fraction, like that of cabbage (Housley and Bentley, 1956), has auxin activity. Chromatography in ammoniacal isopropanol results in either one or several zones of growth promotion, two of which have similar R_fs to IAA and IAN. The zone with R_f of IAA, when rechromatographed in the same solvent, can be converted to a substance travelling at the IAN position, and thus it bears a close resemblance to the IAN-precursor system in cabbage. This suggests that the same system may also be present in tomato.

Since IAN can be readily detected on a chromatogram by chromogenic reagents, and in particular by nitrous/nitric acid which is a sensitive spray for

this compound, it should be possible to show whether the nitrile is present on the chromatogram at *Z* (Fig. 1*e* and *g*). Although several attempts were made to detect IAN on chromatograms of the ether and water-soluble fractions, a nitrile reaction was never obtained, not even a transient reaction when heavily loaded chromatograms were used. One may conclude, therefore, that if IAN was present in these excised and seedling tomato roots, it must have been at very low concentration.

It is of interest that Nitsch and Nitsch (1955) think that IAN is present in tomato fruits. If this is true, then the implications of this with respect to the present work call for careful consideration. However, their evidence for the presence of IAN appeared only to be biological activity at the position of this hormone on chromatograms developed in ammoniacal isopropanol and in aqueous hexane. Results of chromogenic sprays are not mentioned, but Nitsch (personal communication) states that no colour could be detected after spraying with ferric chloride/perchloric acid. Further work is necessary before the presence of IAN in tomato can be accepted as fact.

Although there is doubt whether IAN occurs on chromatograms at *Z*, results in Fig. 2*d-f* show that other substances are present, at least after elution of the chromatogram and extraction by sodium bicarbonate solution. Fig. 2*d* indicates that part of the material at *Z* has entered the neutral fraction, while Fig. 2*f* shows that part has remained in the aqueous phase. In view of the technique used for extraction of acidic from neutral material by bicarbonate solution and the fact that the histogram pattern in Fig. 2*d* and *f* is not obtained in the acid fraction (Fig. 2*e*), it would seem probable that mutual contamination of these fractions has not occurred. It follows, therefore, that in Fig. 2*f* activity at the IAN zone results from a water-soluble ether-insoluble substance, and cannot therefore be due to the nitrile: one may also add that it cannot be due to IAN liberated from its water-soluble precursor (Housley and Bentley, loc. cit.), as the precursor does not travel at the position of *Z* on chromatograms developed in *n*-butanol/ammonia. In addition to the active substance in Fig. 2*f*, it is clear that other growth substances have appeared at positions other than *Z* (Fig. 2*d*, Rf 0-0.1 and 0.43-0.54; Fig. 2*e*, Rf 0.24-0.35). These zones will be considered in later discussion.

It has been noted in the preceding section that in addition to *Z*, two other zones (*X* and *Y*) occur on primary chromatograms of the aqueous fraction developed in ammoniacal butanol (Fig. 1*e* and *g*, and 2*a*), whereas in ammoniacal isopropanol there is frequently only one additional zone at the IAA position. Fig. 1*d* and *h* clearly show that the latter zone when rechromatographed in ammoniacal butanol gives rise to *X* and *Y*.

The experiment of Fig. 2*a-c* suggests, with the reservations already noted in the preceding section, that *X* and *Y* are interconvertible; furthermore, it will be noted in Fig. 2*b* and *c* that rechromatography of these two zones resulted in the appearance of *Z* on both chromatograms. From this experiment and that of Fig. 1*d* and *h*, one may suggest that the active material at *Z* is formed from that at *X* and/or *Y*. Taking the system one step farther, it is

quite possible that the active substances in all three zones are interconvertible. Although direct data on this point are not available, it is of interest that rechromatography of the neutral fraction of *Z* in isopropanol/ammonia (Fig. 2*d*) has resulted in a peak of growth promotion with the same *R_f* as IAA, the position where *X* and *Y* would be expected to travel in this solvent.

It would be interesting to know whether activity in zones *X* and *Y* results from one substance, or whether there are two or more active compounds in each zone. However, it is clear from the experiments of Fig. 1 and 2 that the growth promoters in the various active zones are unstable and readily convertible to other compounds following elution or treatment with sodium bicarbonate solution; thus, considerable care will be required in the interpretation of data obtained from further examination of these zones. The only available experiment bearing on this problem is that of Fig. 1*f*. Provided one accepts the interpretation that the active zone at 31 cm. is equivalent to zone *Y* in Fig. 1*e*, then it would appear that there may be at least two and possibly three growth promoters in zone *X*.

Hormones in the ether-soluble fraction. Chromatography of acid and neutral material in this fraction has resulted in zones and patterns of growth promotion which differ from those described in the literature (reviewed by Kefford, 1955); on the other hand, zones of growth inhibition similar to those obtained by other workers are recognizable. In the acid fractions, inhibition at *R_f* 0.6 occurred on certain chromatograms (Fig. 3*a* and *b*) but not on others (Fig. 3*d*); Bennet-Clark and Kefford (1953) obtained inhibition at a similar position on their acid fraction chromatograms of various plant materials, and ascribed it to a substance termed inhibitor- β . Other inhibition occurred on some chromatograms on and near the starting-line (Fig. 3*b*); however, inhibition at this position is not uncommon (see Fig. 1), and in the aqueous fraction it has been noticed that it usually coincides with pigment which is probably partially responsible for it.

While inhibition occurred at *R_f* 0.0-0.1 on some chromatograms, on others growth promotion was present at this position (Fig. 3*c-f* and *h*). Bennet-Clark and Kefford (loc. cit.) ascribed this promotion to a single acidic substance termed accelerator- α (α); however, in the preceding paper (Housley and Bentley, 1956) it is shown that α is probably an ether-soluble neutral substance, and that it may be formed from water-soluble precursors by heat treatment followed by sodium bicarbonate extraction. Although no attempt was made to demonstrate α in the present work, it is quite possible that some was accidentally formed and that promotion in the neutral fraction at *R_f* 0.0-0.1 results from this substance.

It would be of considerable interest if promotion at this position in the neutral fraction of *Z* (Fig. 2*d*) results from α too, for this would suggest that α can be formed from material at *Z*. Of course, it is possible that it may first have to be transformed into one of the compounds that travel at *X* or *Y* before change to α can occur.

Since α is formed as a result of extraction with sodium bicarbonate solution, it is quite possible that it does not occur in the normal plant hormone system, and that it is an artifact resulting from the method of preparation of the extract for chromatography. This interpretation may also be correct for another promoter which occurs in a number of the acid fractions (Fig. 2*e*, Rf 0.24–0.35; Fig. 3*b* and *d*, Rf 0.3–0.4); Fig. 3*b* gives the best estimate of the Rf limits, which are between 0.3 and 0.4. In the aqueous fractions of Fig. 1*a–d* there is little evidence of its presence with the possible exception of the zone at Rf 0.34–0.45 in Fig. 1*b*. This histogram, when compared with those of Fig. 3*b* and *d*, is complicated by uneven chromatogram division; however, the other experiments of Fig. 1 suggest that the zone actually results from the *X* and *Y* promoters which normally travel at approximately Rf 0.5 in the *isopropanol* solvent system.

If the above interpretation is true it would appear, therefore, from Fig. 2*e* that treatment with sodium bicarbonate solution of one of the substances at *Z* on chromatograms of the ether-insoluble fraction results in the formation of an acidic ether-soluble growth promoter. Although this substance appears to be acidic, the zone in Fig. 3*e* at Rf 0.27–0.39 suggests that it may not have been completely extracted by bicarbonate from the 'neutral' fraction. Similar incomplete separation has possibly occurred in Fig. 3*g* (Rf 0.36–0.48) and 3*h* (Rf 0.31–0.52), but these zones could also result from the growth substances of *X* and *Y*, as the ethereal solutions containing the neutral material were not washed.

Growth substances of the *X* and *Y* regions probably occur to some extent on other chromatograms too, particularly those in which extraction with bicarbonate solution was carried out in a manner similar to that of Fig. 3*g* and *h*. Thus in Fig. 3*d* growth between Rf 0.4 and 0.6 may be due to *X* and *Y*, while in Fig. 3*c* these zones may be present at Rf 0.2–0.3 and Rf 0.4–0.5 respectively. It should be mentioned that stimulation in the IAA regions of the chromatograms is not due to IAA itself; this is clearly indicated by chromogenic sprays. It has been pointed out elsewhere (Housley and Bentley, loc. cit) that the present method of plant extraction would cause the destruction of any IAA present, thus one cannot be certain whether this hormone was absent originally in the tomato roots.

In addition to the zones of growth promotion considered in the preceding discussion, there are at least two other zones of promotion on the histograms. In the ether and aqueous fractions there is evidence of a promoter at Rf 0.1–0.2 in Fig. 1*b*, 3*b*, 3*d*, and 3*g*, and possibly it is present in Fig. 3*a* and *e* too. Promotion at a similar position has been obtained on a number of chromatograms of the ethereal and aqueous fractions of cabbage (Housley and Bentley, loc. cit), and quite possibly it results from the same substance. If this is true, then its repeated appearance in the aqueous fraction indicates that it has not been formed as a result of extraction by sodium bicarbonate solution.

The other zone of promotion on the histograms of Fig. 3 occurs at the IAN position. If substances of zones *X* and *Y* were present on these chromato-

grams as has already been suggested, then one would expect to obtain some of the materials of *Z* at this position on at least some and possibly on all the chromatograms. Whether there are other active substances here too is uncertain; however, it is of interest that maize seedling roots contain neutral ether-soluble active material that travels at the position of IAN on comparable chromatograms, and gives no chromogenic reactions with the present spray reagents (unpublished results from this laboratory). Thus, it seems quite possible that growth in this region of the histograms of Fig. 3 results from more than one substance.

It is evident that a hormone system of some complexity is present in tomato roots. Some components of this system are readily interconvertible, while others change to different growth-active compounds under the influence of sodium bicarbonate treatment.

Although nothing is known about the chemical structure of the active materials at present, the chromogenic spray data lead to interesting speculation. It has been noted that the ether-soluble material gave no reaction with sprays on the chromatograms, and that the only reactions obtained were on chromatograms of the aqueous fraction. On these, ferric chloride/perchloric acid had no action while nitrous/nitric acid gave a yellow colour on one occasion (Fig. 1*a*, Rf 0.36–0.48). MeAB produced the most consistent positive results with a yellow colour at the IAA position on *isopropanol* chromatograms (Fig. 1*c*, Rf 0.42–0.48) and a similar yellow at the corresponding position on *n*-butanol chromatograms (Fig. 1*e* and *g*, zone *X*): other colours were pink between zone *X* and the starting-line (Fig. 1*e* and *g*), and on one occasion (Fig. 1*c*) a pale blue at Rf 0.74–0.83 (not resembling IAN).

The lack of chromogenic reactions in the ethereal fraction and (with two of the sprays) in the aqueous fraction suggests that indole compounds were not responsible for inducing growth on the chromatograms of Figs. 1–3. This opinion is strengthened by the positive results obtained with MeAB, and in particular by the yellow coloration which showed the most consistent correlation with biological activity. It has been pointed out that indole compounds usually give a purple colour with this reagent (Stowe and Thimann, 1954).

On the other hand indole compounds may be present at concentrations too low to be detected chromogenically, and the colour reactions obtained from the aqueous fraction may be due to compounds not connected with the native hormone system. There are insufficient data to express a firm opinion; however, it is of interest that studies on physiological ageing in excised tomato roots have led Street (1955) to suspect that a non-indole system may be operating, and the present work would suggest that future studies on this plant material may reveal the existence of such a system.

The authors have pleasure in repeating the acknowledgements made in the preceding paper of this series. They also have pleasure in thanking Dr. H. E. Street for suggesting the problem, and for providing facilities for the growth of the experimental material.

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Cherelle Wilt of Cacao

I. POD DEVELOPMENT AND ITS RELATION TO WILT

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SUMMARY

The growth and development of cacao pods of three different selections was studied in relation to pod loss through cherelle wilt. Pods were observed to be specially liable to wilt at two stages in their development. These periods were called 'first wilt' and 'second wilt' respectively.

First wilt reaches its peak at 7 weeks after pollination and is shown to cease as cell walls are laid down in the endosperm. Second wilt reaches its peak at 10 weeks from pollination and declines in response to greatly increased pod metabolism. Pods which wilt during second wilt have larger embryos and smaller pod stalks than comparable healthy pods.

It is postulated that both types of wilt arise as a result of a lack of hormones produced by the endosperm, causing a decrease in the uptake of water and food materials and thereby bringing on wilt.

This theory is discussed with reference to previous work and to the possible use of growth substances as a control measure.

INTRODUCTION

IN many orchard crops the phenomenon of 'fruit drop', whereby a significant part of the potential crop is lost by abscission of the developing fruit, is familiar. In the cacao tree (*Theobroma cacao* L.), fruit drop does not occur but instead a large part of the potential crop is lost through a process known as 'cherelle wilt' in which the young pods or 'cherelles' wilt and mummify on the tree. The economic significance of this is evident when it is realized that, even in the best-yielding selections, from 70 to 90 per cent. of the cherelles formed wilt and fail to reach maturity.

Research has been carried out into the causes of cherelle wilt for many years, culminating in the detailed work of Humphries (1943-50) who indicated that wilt is associated with competition for inorganic nutrients between developing pods. Voelcker (1937) had suggested that competition between pods was the cause but had been unable to decide which food materials were limiting. It had been generally remarked that wilt was highest at times when the tree was growing vegetatively or 'flushing' and when, therefore, competition for nutrients was great. Explanations other than physiological have been put forward. McLaughlin (1952) claimed that wilt was due to attack by *Phytophthora palmivora* Butl. and Bartolomé (1954) suggested that leaf hoppers might be important.

Up to 1950 most attempts to control wilt had failed. Cope (1940) claimed a reduction in wilt by adding potassium to the soil, but Bartolomé (1951) was

unable to confirm this. McDonald (1933) showed that, in some cases, applications of phosphatic fertilizers had given increased yields, apparently not by reducing incidence of wilt but by increasing the number of cherelles set. Murray (1953) has shown in Trinidad, in an experiment involving the interaction of shade and fertilizers, that increased yields are due to the production of a larger number of cherelles and not to any decrease in the percentage of wilt.

In Colombia, however, Naundorf and Villamil (1950a) claimed a reduction in wilt by spraying cacao pods with 0.2 per cent. naphthoxyacetic acid (NoXA) followed one week later by 0.05 per cent. naphthalene acetic acid (NAA). Later (1950b) they used two sprays of NAA at an interval of a fortnight and showed a reduction in wilt from 99 to 3 per cent. Gardner and Naundorf (1950) state that wilt was reduced from 66 per cent. to zero with one application of either 25 to 50 p.p.m. *p*-chlorophenoxyacetic acid or 50 to 100 p.p.m. of NAA. Posnette (unpublished data) in the Gold Coast used these growth substances but found no decrease in wilt. Murray (1952) in Trinidad repeated the Colombian experiments but could obtain no reduction in wilt with varying concentrations of NAA, NoXA, 2:4-dichlorophenoxyacetic acid (2:4D), 2:4-dichlorophenoxypropionic acid or 2:4:5-trichlorophenoxyacetic acid. The present worker (1954) repeated the experiments with NAA and 2:4D but did not succeed in reducing wilt.

With these various results in mind, it was decided to reinvestigate the factors causing wilt to determine whether wilting has a hormone basis as has fruit drop in other crops, and whether from a theoretical standpoint any success could be expected by spraying with growth substances.

EXPERIMENTAL METHODS

For the study of fruit development cacao has an advantage over many crops in that pods are set throughout most of the year. Thus by carrying out pollinations at different times throughout the season the effect of environment on pod growth and development can be eliminated to a great extent. Fruits at various stages of development can be gathered at the same time. As already noted the incidence of wilt is greatly influenced by leaf flushing, and it is important that this factor should be allowed for when attempting to relate wilt incidence to pod development.

Vyvyan (1946) working with apples devised a dropping index which overcomes the difficulty of deciding whether drop should be related to fruit at the beginning, middle, or end of the period concerned. This index has been adapted so as to give a figure (Q) for rate of wilting of cacao pods.

$$Q = \frac{\log x_1 - \log x_2}{t_2 - t_1},$$

where x_1 and x_2 are the number of healthy (unwilted) pods on the tree at times t_1 and t_2 respectively. The use of this index is more precise than expressing the absolute numbers of wilted pods (e.g. Hewison and Ababio, 1929)

because numerically there are always, throughout a season, more small pods than large one, because of the loss of pods due to wilt.

Six 10-year-old trees of each of T60 (Upper Amazon), T9 (Trinitario), and A139 (Amelonado) selections were chosen and several hundred flowers were hand-pollinated from February to July. After pollination the length of each pod was measured weekly and the age and size at which it wilted was noted.

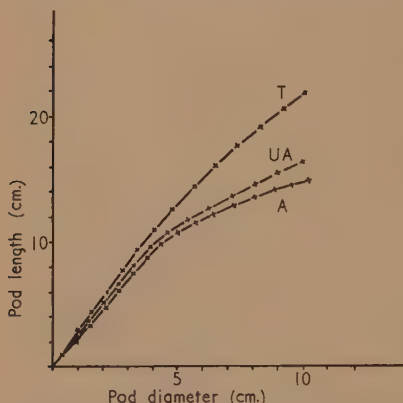


FIG. 1. Relation of pod length to pod diameter during development, Trinitario (T), Upper Amazon (UA), and Amelonado (A).

ratio of length to breadth remains constant. Fig. 1 shows that this ratio is constant until the pod reaches a length beyond which wilting does not occur. Hence increase in pod length has been used in the present experiments as a measure of growth.

RESULTS

Pod development. From the emergence of the flower bud through the bark to flower-opening occupies approximately 30 days. Failure of fertilization at the time of flower-opening stops ovary growth and the flower abscises usually after 1 day (Fig. 2). This, it may be noted, is the only stage in the development of a pod at which abscission occurs. Post-fertilization growth assumes an S-shaped curve.

There were found to be two distinct periods of development from fertilization to harvest. They are:

Period I. The young pod begins to enlarge in conjunction with the ovules. Growth in length is slow up to about 40 days, then it becomes rapid and reaches a maximum at about 75 days, corresponding to an Amelonado pod of 11 cm. long. It is at this time that the pod begins to swell and it is between 80 and 120 days that growth is most rapid if the criterion of weight is used. The ovules grow in a similar manner to the pod. They are at first easily detachable from the pulp, but as time goes on they become more firmly attached to it.

The date of wilting was taken as the last date on which an increase in length was recorded. This was done because a preliminary experiment had shown that pods cease to elongate about 1 week before visible symptoms of wilting appear. Pods damaged by *Phytophthora* or by rodents were discarded from the experiments.

Pod measurement. Hunter and Waters (1928) showed that the specific gravity of a cacao pod remains constant at 0.98 throughout growth until ripening begins. Thus volume can be employed as the measure of growth. Since the cacao pod approximates to a prolate spheroid during its growing period, linear dimensions can be used to estimate volume, provided the

The zygote does not divide until about 40 to 50 days after pollination (Cheesman, 1927) and even after that growth of the embryo is slow.

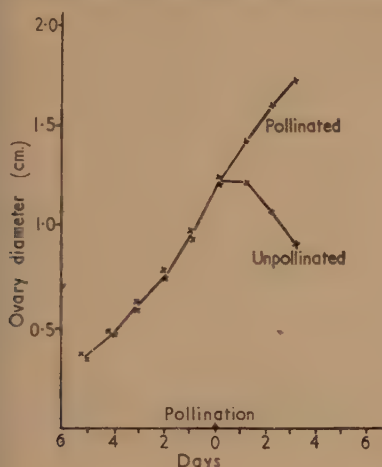


FIG. 2.

FIG. 2. Growth of pollinated and unpollinated ovaries. The unpollinated flower never remains on the tree for more than three days, normally only one or two days.

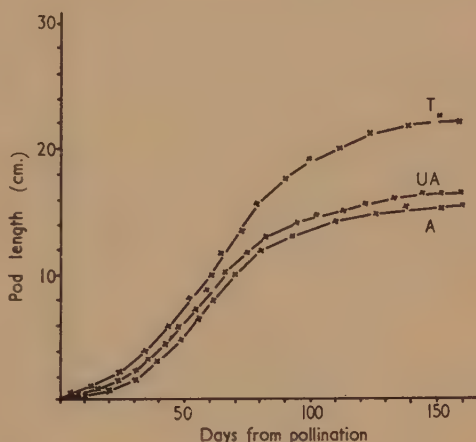


FIG. 3.

FIG. 3. Growth of pods of three selections, Trinitario (T), Upper Amazon (UA), and Amelonado (A).

Period II. This period begins at about 85 days after pollination. Pod and ovule growth slow down at the expense of embryo growth. The embryo grows from 0.2 cm. in length after 85 days to over 3 cm. after 140 days, when it completely fills the bean. Before embryo growth becomes rapid the ovule becomes filled with jelly-like endosperm. As the embryo develops this is consumed until at 140 days the endosperm has vanished. When embryo growth ceases there is no resumption of pod growth as in many fruits: ripening begins almost at once.

Humphries (1943) has described a period of rapid accumulation of fat from 87 to 143 days which corresponds with this second period.

Development of different selections. Fig. 3 shows that the growth curves of the pods borne by the three different selections used in these experiments are very similar. For length, maximum growth rate is reached at about 75 days in each selection.

Development of the pod stalk. When the pod is young the stalk by which it is attached is long and slender. As the pod grows the stalk increases in width and to some extent contracts in length. Fig. 4 shows a variable relation between stalk length and pod length but a close linear relation between the diameter of the stalk and length of the pod.

Pod development and wilt. The relationship between susceptibility to wilt and age of 150 hand-pollinated pods of each selection is shown in Fig. 5.

There are two peaks of wilting in each variety. The first and larger peak occurs at about 50 days and the second at 70 days. There is an increase in

the incidence of wilt from the time of pollination to the first peak, a decrease till about 60 days when there is a rise to the second peak, and a fall to zero at 95–100 days.

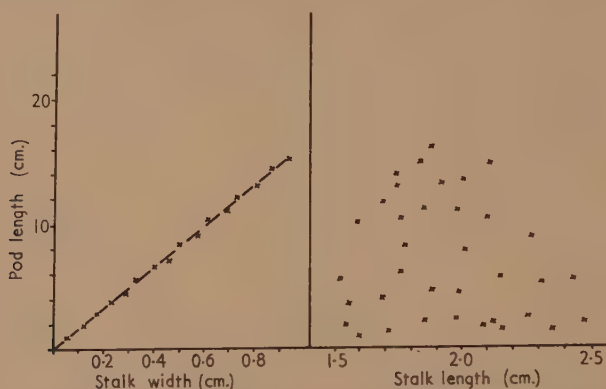


FIG. 4. Relation of pod growth to stalk growth. Stalk length is very variable.

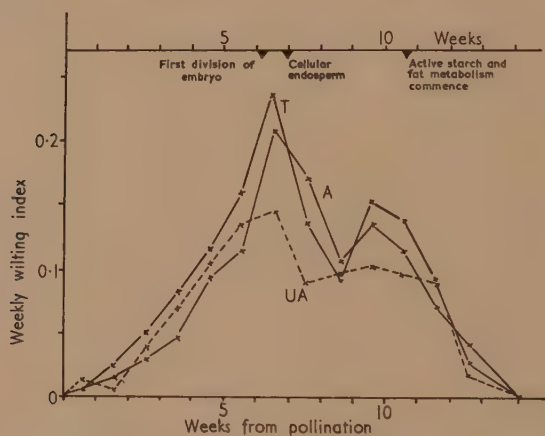


FIG. 5. Wilting index for three cacao selections, Trinitario (T), Amelonado (A), and Amazon (UA), during development. The upper diagram relates the peak of wilting to pod metabolism.

This second peak has not been reported before and unless large numbers of hand-pollinated pods are used it may be easy to overlook it. It is less well marked in the Upper Amazon than in the other two selections but is still present. There is no high peak during the first week as found by Hewison and Ababio (1929).

From Table I it can be seen that the wilting index over the whole period of the experiment varies for each selection, but the variability within trees of one selection is so high that it is not possible to say that any one selection is more susceptible to cherelle wilt than another.

Physical differences between healthy and wilting pods. Since two distinct periods of wilt were noticed, investigations were carried out on a large

TABLE I*

Pod, ovule, and embryo length (in cm.) of Amelonado (A), Upper Amazon (UA), and Trinitario (T) selections in relation to weekly wilting index.

Weeks from pollination	Pod length			Ovule length			Embryo length			Weekly wilting index		
	A	UA	T	A	UA	T	A	UA	T	A	UA	T
0—	0.53	0.55	0.55	0.03	0.03	0.04	0.007	0.012	0.006
2—	1.85	2.15	2.81	0.10	0.12	0.13	0.029	0.039	0.048
4—	3.62	4.14	5.59	0.26	0.30	0.35	0.094	0.104	0.113
6—	5.16	5.91	8.13	0.41	0.47	0.51	first division of zygote			0.209	0.145	0.236
8—	7.01	8.34	11.39	0.80	0.91	0.13				0.105	0.096	0.091
9—	8.53	9.82	13.40	1.11	1.27	1.41	0.16	0.18	0.17	0.135	0.101	0.154
10—	9.92	11.31	15.52	1.44	1.65	1.83	0.26	0.28	0.28	0.115	0.096	0.139
12—	12.18	13.82	18.80	2.01	2.30	2.44	0.51	0.55	0.52	0.004	0.016	0.024
14—	13.31	15.20	20.95	2.34	2.67	2.73	1.01	1.21	1.12	Wilting ceases		
16—	13.94	15.92	21.77	2.48	2.83	2.89	1.71	2.02	1.94
18—	14.31	16.22	22.23	2.58	2.93	3.00	2.41	2.89	2.75	Ripening begins		

* Data for intermediate weeks omitted to reduce length of table (Editor).

number of Amelonado pods to see if both types of wilt showed similar characteristics.

In the field no difference was observed between the manner in which wilting occurred. In both, pods ceased to grow about 1 week before yellowing occurred, followed by browning and shrivelling. In young trees it was noticed that very few pods wilted after attaining a length of 7 to 8 cm. and that it was in mature trees that the larger pods wilted.

Comparisons were made between healthy and wilted pods in regard to length:diameter ratios, stalk length and width, pod volume and weight, number of ovules, and size of ovules and embryos. For the first period of wilt or 'first wilt' no differences could be observed between healthy and wilting pods.

In the second period of wilt or 'second wilt' two differences were found between healthy and wilting pods. Firstly, pods which wilt after 8 weeks old have comparatively narrow stalks. Fig. 6 shows that the ratio of

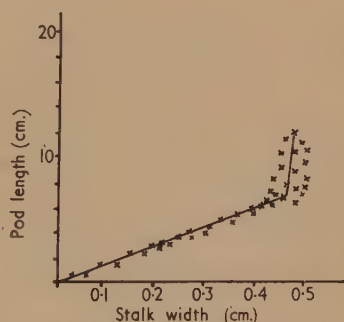


FIG. 6. Failure of pod stalk growth during second wilt.

pod length to stalk width increases greatly for wilting pods longer than 8 cm. It would appear that in a pod about to wilt in the second period, the stalk ceases growing while the pod is about 8 cm. long. Secondly, wilting pods longer than 8 cm. were found to have larger embryos than had comparable healthy pods (Fig. 7). Five embryos were taken from each of 100 pods and their length measured. As far as possible only pods just beginning to wilt were used since wilting pods soon begin to decrease in length. Pod growth slows down or stops about 1 week before symptoms of wilting appear, hence 1 week's growth increment was added to the figures for the wilted pods which were being examined for embryo size. This adjusted figure was used in Fig. 7.

Stalk development in relation to wilt. Humphries (1944) postulated that up to about 75 days from pollination the pod relies mainly on the xylem for its supply of mineral nutrients and water. When, after 75 days, phloem transport becomes active, according to Humphries, the pod becomes less sensitive to competition for minerals, and less liable to wilt. In view of this theory that transport of minerals in the phloem is of minor importance in pods up to 75 days old and that wilt ceases after this because of increased phloem transport,

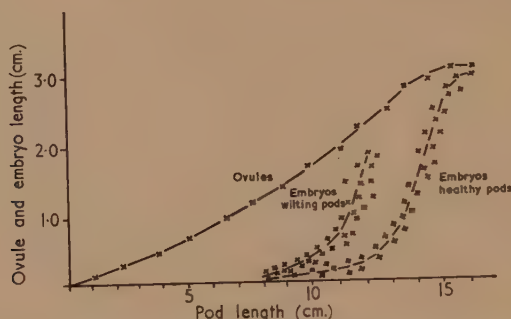


FIG. 7. Data showing the enlarged embryos found in pods during second wilt.

experiments were carried out to find the effect of ringing of pod stalks on pod growth and to assess the relative proportions of xylem and phloem in these stalks during growth of the pod.

Cacao pod stalks in transverse section are seen to have a central pith enclosed by a cylinder of xylem. The phloem consists of broadly triangular areas alternating with numerous wide primary medullary rays. The phloem breaks away readily from the xylem and in the field ringing is easily carried out.

Ninety Amelonado pods were measured. Thirty of these were left as controls, 30 were completely ring-barked, and on the remaining 30 only half the circumference of the bark was removed. The treated stalks were smeared with wax to prevent water loss through the exposed surfaces. Microscopic examination showed that in the barked specimens phloem only was removed and that the xylem remained intact.

Where the stalks were completely ringed, pod growth stopped very rapidly and all the pods had wilted after twelve days (Table II). Pods on the half-ringed stalks slowed down slightly in growth as compared with controls but only one had wilted after 11 days. No control pods had wilted. Even in the smallest pods removal of the phloem caused decreased growth, indicating that there is always a demand for nutrients carried through the phloem. Differences between growth of controls and half-ringed pods are significant at 5 per cent.

It is of interest that fully ringed pods can wilt when 15 cm. long. That they do not do so under natural conditions implies that as long as the phloem

TABLE II

Average daily increase in pod length (cm.) for 12 days after ringing

	Pods 0-4.9 cm.	Pods 5-15 cm.
Control	0.129 ± 0.006	0.135 ± 0.006
Half ringed	0.101 ± 0.003	0.105 ± 0.003
Fully ringed	Wilted	Wilted

is functioning properly, wilting will not occur and that the nutritional requirements of the pod can always be met. Examination of the amounts of phloem and xylem in pod stalks indicates an almost constant proportion of the two types of conducting strands during development. Fig. 8 shows the

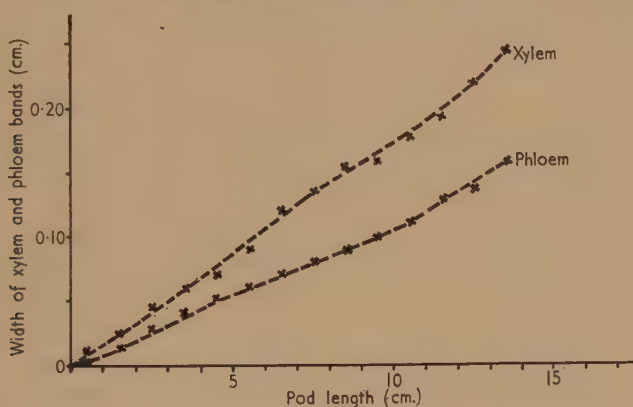


FIG. 8. Development of xylem and phloem in pod stalks.

ratio of the width of the bands of xylem and phloem with increasing pod size. Both xylem and phloem increase steadily and there is no sudden increase in the proportion of phloem after 75 days (or 11 cm.). It is, of course, not necessary to have a sudden increase of phloem at 75 days for there to be increased transport in the phloem at that time.

DISCUSSION

The present experiments have shown that the tendency for a pod to wilt is dependent on the stage of development of the pod. This does not mean that wilt is not affected by factors such as flushing or drought but that environmental conditions being equal, a pod is more liable to wilt at certain stages of development than at others. The peak of wilting in the first week after pollination reported by Hewison and Ababio (1929) has not been seen here. In the present experiments wilting was lowest in the first week. The high figures they obtained for the first week were probably due to inclusion of the drop of unpollinated flowers.

Rounce and Smart (1928) showed that wilt was most prevalent about 50 days from pollination and suggested that this was a result of the first division

of the zygote. Voelcker (1937), too, found a danger period at 50 days old but suggested this was due to a physiological crisis as a result of heavy setting and flushing at that time. In an attempt to eliminate the effect of environment on wilt he carried out hand-pollinations over a period of several months and concluded that physiological phenomena within the tree and not within the cherelle caused wilting. A re-examination of figures obtained by Voelcker in Trinidad and in Nigeria and a transference of them from a numerical to a Wilting Index basis shows, however, approximately the same two peaks as found here (Table III.) Since it is not known whether Voelcker estimated the date of wilting of pods in the same way as in the present experiments, and since he grouped together all losses up to 15 days from pollination and did not separate losses due to failure of fertilization from loss due to cherelle wilt, it is difficult to compare the two sets of results very accurately.

TABLE III

Weekly wilting index of cacao pods in relation to pod development. Data after Voelcker (1937)

Weeks from pollination	Wilting Index	
	Nigeria	Trinidad
2—	0.064	0.101
3—	0.062	0.074
4—	0.093	0.097
5—	0.124	0.099
6—	0.117	0.104
7—	0.109	0.087
8—	0.0743	0.051
9—	0.049	0.077
10—	0.064	0.110
11—	0.027	0.064
12—	0.014	0.069

Since there are these two periods it will be necessary to consider what are the causal factors and how wilt is linked with pod development and metabolism. The two types of wilt have been shown to have different physical characteristics and they may be caused by quite separate factors.

The end of the first period of wilt coincides with the formation of cell walls in the endosperm as determined by Cheesman (1927). The first division of the fertilized egg was estimated by Cheesman to occur between 40 and 50 days after pollination. The first cell walls in the endosperm appear not less than 50 days from pollination.

'First' wilt passes its peak at about 50 days and thus would seem to coincide more closely with the formation of cellular endosperm than with the first division of the zygote. It has been shown by Harrold (1935) for peaches, and more recently by Luckwill (1953) for apples, that the development of cellular endosperm is the signal for the end of the first period of fruit drop. Luckwill showed that the endosperm is probably the site of hormone production in

the seed and that when cell walls are laid down an impetus is given for increased supplies of hormone for fruit growth. It has been shown in maize and in other cereals that the endosperm is the site of hormone formation (Avery, Creighton, and Shalucha, 1940).

From this it would appear likely that in cacao as cell walls are laid down in the endosperm, hormone production increases. That this coincides with the end of the first period of wilt suggests that hormone levels are involved in the mechanism of wilt. It is postulated that when the pod reaches 7 weeks old its susceptibility to wilt decreases because of an increase in the amount of hormone available.

The end of the second peak of wilt at about 11 to 12 weeks old occurs at the same time as greatly increased active fat and starch metabolism commencing at about 75 days old. Luckwill (1953) showed that in apples, rapid starch formation coincides with the end of June drop and at the same time there is a second peak in the production of hormone.

During second wilt it was shown that stalk development is slowed down. Nitsch (1952) has described several instances where vascular development of the fruit stalk is controlled by hormones. Jacobs (1951), studying the regeneration of interrupted vascular strands, established a correlation between the amount of growth substance applied and the number of regenerated vascular bundles. Thus the decrease in stalk growth may be due to a lack of hormone.

There would appear to be an analogy between wilt in cacao and drop in other fruits. The 'first' and 'June' drops of apples seem equivalent to the 'first' and 'second' wilts of cacao. In apples and in cacao the end of first drop and of first wilt respectively occurs at the same time as the formation of cell walls in the endosperm. During June drop of apples the endosperm is consumed and hormone levels are low while the embryo grows rapidly. This is almost the same pattern as for second wilt of cacao. Embryo growth is increasing rapidly and in wilted pods the embryo is proportionately larger than in healthy pods. These enlarged embryos must consume more endosperm than do normal embryos and hence hormone levels at this point will be low.

We can now compare this new theory in the light of previous knowledge obtained by Humphries. The information obtained during the course of the present experiments is largely compatible with that work. While it had been suggested here that cherelle wilt is under the control of hormones produced in the pod, this is not to deny that it is a lack of nutrients which in the final analysis causes a pod to wilt. Hormones are known to control water and nutrient uptake (Ketelapper, 1953; Mitchell and Marth, 1950) and it is probable that a lack of hormone at critical stages in the development of the pod is responsible for wilt. Pods at 7 and at 10 weeks after pollination are most likely to wilt because they appear to have least hormone.

The only point in which the present work differs from that of Humphries is in the question of transport of food materials in the phloem. Ringing the

stalks of the pods does not provide a direct test of the theory that up to 75 days mineral transport is mainly via the phloem, since it must also interfere with the transport of nitrogen and sugars to the developing pod. Humphries postulated that up till 75 days there is little demand for minerals in the phloem, but it seems likely that if pods wilt before this time because of insufficient mineral nutrients there must be a demand for them. This present series of experiments suggests instead that wilt ceases after 12 weeks because of increased supplies of hormones and consequently the developing pod is better able to take up water and nutrients.

These experiments do not prove conclusively that cherelle wilt is under the control of hormones produced by the endosperm but they do provide circumstantial evidence for such an hypothesis. Such an hypothesis has been almost completely proven for other fruit crops (Nitsch, 1952) and it is logical to expect its validity in cacao. The dissimilarity between the phenomenon in cacao and in other crops lies in the absence of abscission in cacao. To test whether there might be an inherent, but inactive, abscission mechanism in cacao, pods were cut off leaving the stalk attached to the tree, according to the method described by Barlow (1948). Although pods of all sizes were used there was no abscission even after 4 weeks, when the stalks began to blacken and shrivel up. This is in contrast to most fruit trees, where defruited stalks absciss within 1 week. When cacao flowers absciss they do so at a definite abscission zone and although this zone remains visible during subsequent development of the pod it does not function after fertilization. It may be noted that in *Theobroma bicolor*, pods are shed just before ripening, involving a definite abscission zone.

From the above results and considerations it would seem possible that growth substances applied to the cacao pod might help to prevent wilt. Only pods at about 7 and 10 weeks from pollination would be expected to benefit, because of deficiency of hormones at such times, and growth substances applied to pods at other stages may have no effect or may even be deleterious.

In most fruit crops timing of growth substance sprays is all important but is relatively easy to determine. In cacao the problem of the right time for spraying becomes more difficult as there are pods of all different sizes present on the tree at any time.

Experiments have been carried out to apply the theoretical deductions from the above results to the practical control of cherelle wilt. Increased production of ripe pods can only be brought about by increased food supplies to the tree or by a more efficient use of existing supplies. Growth substance sprays are being used in an effort to increase yield at certain times either directly through a reduction in wilt, through selective thinning of flowers and pods, or through the control of 'flushing'. These results will be presented in a later publication.

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The Uptake of Calcium and Strontium by Plants from Soils and Nutrient Solutions

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Received 4 November 1955

SUMMARY

Plants have been grown in soils and nutrient solutions containing different Ca/Sr ratios to find out whether there is any biological discrimination between calcium and strontium.

When tomato plants are grown in nutrient solutions containing molar Ca/Sr ratios from 2/1 to 4,000/1, the shoots do not distinguish between the elements, but the roots absorb strontium preferentially at low concentrations of this element.

Since ion exchange resins show the same phenomenon, though to a less marked extent, this is believed to be a physico-chemical rather than a biological effect.

No chemical reagent has been found which can extract calcium and strontium from soils in the same ratio as plants.

Ammonium acetate extraction may give misleading results for the plant-available Ca/Sr ratios in the soil. Data for the Ca/Sr ratios in barley and lucerne grown on soils containing ratios from 3/1 to 650/1 emphasize this point.

STRONTIUM is an element which is universally present in soils but its uptake by plants has been but little studied until recent years. The occurrence of two long-lived isotopes of this element among the fission products of uranium has stimulated considerable research into its behaviour in soils and plants (Rediske and Selders, 1953; Larson, Olafson, Neel, and Steen, 1953; Neel *et al.*, 1953; Nishita, Kowalensky, and Larson, 1954; Romney, Rhoads, and Larson, 1954; Epstein and Leggett, 1954; Long, Teubner, Wittner, and Tukey, 1954; Bowen and Dymond, 1955). It has been established that, unlike other fission products, the element is only partially absorbed by soils in a form not available to plants (Nishita *et al.*, 1954; Newbold and Russell, personal communication). Other workers have shown that strontium is taken up into the aerial parts of plants roughly thirty times more readily than any other fission product (Larson *et al.*, 1953; Jacobson and Overstreet, 1948) and that strontium applied to leaves is readily translocated into the cells (Wolf and Cesare, 1952). Animals may absorb radioactive strontium by feeding on contaminated vegetation (Cross, Taylor, Lee, and Watson, 1953) and the element is then concentrated in bones where it may constitute a serious health hazard (Hamilton, 1947). The permissible body burdens for man of 54-day Sr^{89} and 28-year Sr^{90} have been fixed as 2 and

$1\mu\text{C}$ respectively, and a maximum permissible level of contamination for pasture herbage has been suggested as $5 \times 10^{-6}\mu\text{C Sr}^{90}/\text{g}$. (Chamberlain, Loutit, Martin, and Russell, 1955). Since the total fission of one gram of U^{235} gives rise to $3.7 \times 10^8\mu\text{C Sr}^{89}$ and $1.5 \times 10^6\mu\text{C Sr}^{90}$, it is clear that the most careful precautions should be taken to minimize the contamination of the globe by fission-product strontium which will inevitably enter the biotic cycle.

Strontium is well known to follow calcium closely in plants and soils (Collander, 1941; Mitchell, 1937), but recently some American workers have claimed that plants differentiate against strontium by a factor of approximately three (Menzel, 1954; Comar, 1955). Some analyses of plants growing in native English soils, however, indicated a slight preferential uptake of strontium except from a few rare soils containing large amounts of strontium sulphate (Bowen and Dymond, 1955). The object of the present work was to determine the extent of the biological differentiation between calcium and strontium, and for this purpose plants were grown in soils and nutrient solutions with different Ca/Sr ratios and subsequently analysed for the two elements.

METHODS

Nutrient solutions. Tomato plants (*Lycopersicum esculentum*) were germinated in the dark on cheese cloth saturated with calcium-free nutrient solution. They were then grown for 28 days in continuously aerated nutrient solutions in 'polystyrene' containers. The nutrient solutions contained, per litre, 6 mM (Ca+Sr) as nitrate, 5 mM KNO_3 , 2 mM MgSO_4 , 1 mM KH_2PO_4 , 1 mM $(\text{NH}_4)_2\text{HPO}_4$, 0.1 mM ferric citrate, with added trace elements 1 p.p.m. Zn, 0.5 p.p.m. Mn, 0.5 p.p.m. B, 0.1 p.p.m. Cu and 0.01 p.p.m. Mo. All chemicals used were Analar grade. The molarities of calcium and strontium stock solutions were checked by precipitation as oxalate followed by permanganate titration. Eight Ca/Sr ratios were used varying from 2-4,000. The initial pH of the full nutrient solution was 7.0, but this fell to 6.1 after a week's growth. Solutions were replaced weekly. The tomato plants all looked healthy, though during the last week of growth several of them were attacked by a leaf-mosaic virus.

Soils. Barley (*Hordeum vulgare*) and lucerne (*Medicago sativa*) were germinated and grown for 56 days in 1,100 g. calcareous, sandy soil containing different amounts of added strontium sulphate. Samples of the soils were extracted with various different reagents and the extractant finally chosen was ammonium acetate at pH 9. Two replicate 100 g. portions of soil were shaken for four hours with 200 ml. of this reagent, centrifuged off, and 25 ml. aliquots of the supernatant were taken for analysis. Five soils were used, and the Ca/Sr ratios in the soil extracts varied from 3 to 650.

Analytical methods. Plant roots were given two 10-second rinses in distilled water: shoots were analysed direct. The tissues were dried in an oven at 110°C ., weighed and dry-ashed in silica dishes in a muffle furnace at

500° C. The ashed tissues were powdered in an agate mortar and stored in 'polythene' containers.

Soil extracts were treated with ammonium oxalate solution at pH 5-6. It had previously been established by tracer techniques that only about 0.2 per cent. of the strontium present remains in the supernatant and washings from the oxalate precipitate. Alkali metals, aluminium, phosphate, and other contaminants are removed by this procedure.

Calcium was determined by flame photometry and strontium by activation analysis, using methods which have already been described (Bowen and Dymond, 1955; Harrison and Raymond, 1955). These analyses were accurate to at least ± 5 per cent.

The chemical reagents and seeds used were tested for traces of strontium, using the activation method, with the following results:

	Amount used	$\mu\text{g Sr}$
Ca (NO ₃) ₂ used in 1 litre nutrient solution	6 mM	128
Other constituents in 1 litre nutrient solution	1 litre	3.5
Tomato seed	1	0.015
Soil poorest in strontium	100 g.	50
Ammonium acetate extractant	200 ml.	0.8
Ammonium oxalate reagent	10 ml.	<0.1
Barley seed	1	0.008
Lucerne seed	1	0.002

In all cases the reagent and seed blanks were less than 3 per cent.

RESULTS

Nutrient solutions. Tomato roots and shoots were analysed separately. The mean dry-weight of 64 roots was 0.0304 g. with standard deviation ± 0.0130 g. and of 64 shoots 0.206 ± 0.096 g. The mean dry-weight of the 8 plants in the 8 different nutrient solutions did not differ significantly from these grand means, in agreement with earlier observations for cereal grasses (Scharrer and Schropp, 1937). No symptoms of strontium toxicity were observed (Hurd-Karrer, 1937).

Table I gives the mean calcium and strontium contents of the plants grown in the different culture solutions, together with the amounts of calcium and strontium supplied. Only about 0.6 per cent. of the total (Ca+Sr) supplied was absorbed by the plants, and roughly 95 per cent. of this was translocated to the shoots. All Ca/Sr ratios are atom/atom.

The data of Table I are plotted on a log/log scale in Fig. 1. Here the dotted line is the theoretical curve for the case of no discrimination between the two elements, and it is clear that there is practically no discrimination in the shoots over the whole range of ratios studied. However, the roots do appear to discriminate in favour of strontium at low concentrations of this element, and it is noteworthy that other workers have remarked on the great affinity of strontium for roots (Jacobson and Overstreet, 1948; Rediske and Selders, 1953).

TABLE I
Competitive uptake of Ca and Sr by
tomato roots and shoots from nutrient media

Solu- tion	meqs Ca supplied	meqs Sr supplied	meqs Ca/gm root	meqs Sr/gm root	meqs Ca/gm shoot	meqs Sr/gm shoot	Ca/Sr in root	Ca/Sr in shoot	Ca/Sr supplied
1	16	8	202	98.7	554	223	2.04	2.49	2
2	20	4	262	60.0	709	121	4.36	5.86	5
3	22.86	1.143	245	17.7	814	34.6	13.9	23.5	20
4	23.53	0.472	334	12.7	769	14.9	26.3	51.7	50
5	23.76	0.2376	397	6.32	722	7.06	62.9	102	100
6	23.95	0.4784	283	1.44	659	1.01	196	655	500
7	23.98	0.02398	352	0.826	785	0.728	425	1,080	1,000
8	24	0.00584	300	0.440	734	0.187	681	3,935	4,150

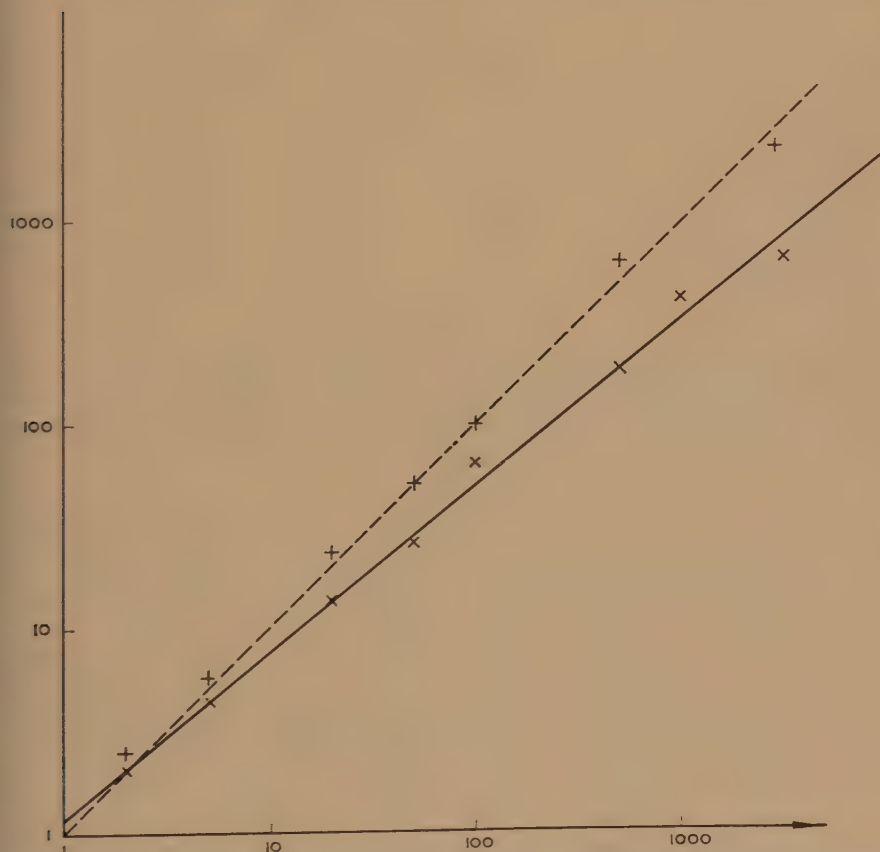


FIG. 1. Competitive uptake of calcium and strontium from nutrient solutions by tomato roots (X) and shoots (+). Ca/Sr ratios in plant shown as ordinates and Ca/Sr ratios in solution as abscissae.

Soils. Only shoots were analysed because of the difficulty of washing the roots free from soil. The mean dry-weight of barley shoots was 1.86 g. with

standard deviation ± 0.97 and that of lucerne shoots 0.675 ± 0.370 g. There were no significant differences in the dry weights of plants grown at the different levels of strontium used here.

There was some difficulty in finding a reagent which would extract calcium and strontium from the soils used in approximately the same ratio as the plants. The soils contained much free calcium carbonate with a Ca/Sr ratio of about 1,000/1, so that acid extractants which dissolved this carbonate gave very misleading results for the 'plant available' Ca/Sr ratio. Some typical ratios extracted from the same soil by different reagents are shown in Table II.

TABLE II

Amounts of Ca and Sr extracted from a single soil by different reagents

Extractant	pH	meqs Ca/ 100 gm soil	meqs Sr/ 100 gm soil	Ca/Sr
Nitric acid	0.97	168	166	1,010
Acetic acid	1.90	161	155	1,040
Ammonium acetate buffer	5.95	35.6	47.0	760
Ammonium malate buffer	5.95	47.0	36.8	1,280
Carbonic acid	3.94	5.52	29.1	190
Boric acid	4.75	6.72	27.0	250
Ammonium acetate/ammonia	9.00	0.373	7.48	50
Ammonium chloride/ammonia	9.00	0.400	7.22	55
Barley (3 plants)	0.090	1.62	56
Lucerne (3 plants)	0.090	1.84	49

The last two reagents in this table were the only two which extracted about the same total amount of calcium from the soils as the plants and they gave the only reasonable values for plant-available Ca/Sr ratios. Ammonium acetate at pH 9.0 was used as a soil extractant to obtain the results of Table III.

TABLE III

Competitive uptake of Ca and Sr by barley and lucerne from soils

Soil	meqs Ca/100 gm soil	meqs Sr/100 gm soil	meqs Ca/g. barley	meqs Sr/g. barley	meqs Ca/g. lucerne	meqs Sr/g. lucerne	Ca/Sr soil extract	Ca/Sr barley	Ca/Sr lucerne
1	444	135	183	14.4	503	61.0	3.28	12.7	8.28
2	415	93.0	202	13.2	709	46.6	4.46	15.3	15.2
3	353	35.7	127	4.05	676	30.0	9.89	31.4	22.5
4	373	7.48	145	2.60	769	15.8	49.8	55.8	48.6
5	355	0.54	172	0.494	510	1.31	656	349	390

These results are plotted on a log/log scale in Fig. 2. They agree qualitatively with the results of a survey of Ca/Sr ratios in native plants and soils, in that with high ratios in the soil Sr appears to be preferred in the plant to calcium, whereas at low ratios it is rejected (Bowen and Dymond, 1955). There is, however, an obvious discrepancy between the soil and nutrient solution experiments which remains to be cleared up.

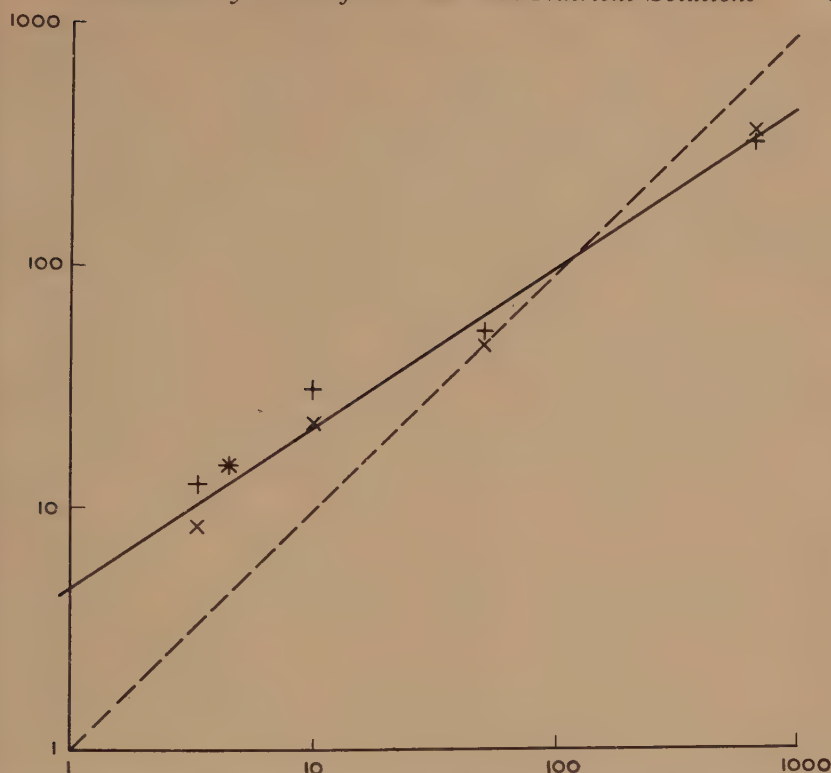


FIG. 2. Competitive uptake of calcium and strontium from soils by shoots of barley (+) and lucerne (X). The dotted line is the theoretical curve for no discrimination. Ca/Sr ratios in plant as ordinates and in the soil extract as abscissae.

DISCUSSION

Before we discuss the results reported above, we should draw attention to the work of Menzel (Menzel, 1954). This author regarded it as axiomatic that the Ca/Sr ratio in a plant should be directly proportional to the available ratio in the soil, i.e.

$$\text{Ca plant/Sr plant} = K(\text{Ca soil/Sr soil}) \quad (1)$$

where the available ratio in the soil is considered. This equation is unlikely to hold in view of the researches of Vanselow (1932) and other workers on base exchange systems, who advocate an empirical relation of the form:

$$\text{Ca plant/Sr plant} = K (\text{Ca soil/Sr soil})^m$$

whence

$$\log (\text{Ca plant/Sr plant}) = m \log (\text{Ca soil/Sr soil}) + \log K \quad (2)$$

which, unlike equation 1, fits the data of Fig. 2, with $m = 0.678$, $K = 4.55$. Both equations fit the tomato shoot data of Fig. 1, with $m = 1$, $K = 1$, but only equation 2 fits the tomato root results with $m = 1.05$, $K = 1.20$. We conclude that equation 1 has neither experimental nor theoretical support.

Menzel claimed that plants discriminate against strontium in soils by a factor of about 2.5 ($K = 2.5$ in equation 1). He added Sr^{89} to a soil of low-calcium content and determined the Ca and Sr^{89} contents of cowpeas (*Vigna sinensis*) grown on the soil. However, he did not determine what fraction of the added Sr^{89} was absorbed by the soil in a form not available to plants, and several workers have shown that soils can absorb a considerable percentage of strontium in this way (Nishita *et al.*, 1954; Newbold and Russell, personal communication). The results of our experiment with nutrient solutions indicate that tomato shoots, at least, do not discriminate between calcium and strontium. Menzel's work gives an overall figure of discrimination by plants against uptake of soluble strontium added to a particular soil, but cannot be accepted as evidence for discrimination by plants against the element: the discrimination is most likely to be an effect of the soil.

The discrepancy between our results in soils and nutrient solutions must also be an effect of soil, or soil-extraction. The results reported above show that ammonium acetate is not a satisfactory reagent for obtaining ratios of Ca and Sr in soils available to plants. It is hardly surprising that no chemical reagent is known which can extract the same ratio from a soil as a plant. Our soil was a peculiar one and it would be dangerous to generalize from it to other soils, but it is clear that there may be other cases where the ammonium acetate extraction procedure is unreliable. However, ammonium acetate has been widely used to determine exchangeable calcium in soils and its reliability in this connexion has recently been confirmed by a method of isotope-exchange (Blume and Smith, 1954).

TABLE IV

Competitive uptake of Ca and Sr by Zeo-Karb 225 resin from solutions

Solution	mM Ca supplied	mM Sr supplied	μM Ca taken up	μM Sr taken up	Ca/Sr supplied	Ca/Sr taken up
1	8.00	4.00	136	61.9	2	2.20
2	10.00	2.00	185	31.6	5	5.86
3	11.42	0.576	188	9.58	19.5	19.6
4	11.76	0.239	190	3.53	49.2	53.8
5	11.88	0.120	192	1.82	99.1	105
6	11.97	0.0276	177	0.434	469	408
7	11.98	0.0159	180	0.273	816	660
8	12.00	0.00685	190	0.120	1,900	1,580

Finally we should offer an interpretation of the results from tomato roots which represent a true discrimination on the part of the plant. Is this discrimination biological or physico-chemical? If biological, it would imply a definite physiological need for strontium, and should be tested by growing plants in solutions containing even smaller traces of the element than we have used. Unfortunately even 'Spec. Pure' calcium carbonate contains about 50 p.p.m. strontium as an impurity and further purification would be difficult. In any case, it seems more likely that the discrimination is purely physico-

chemical, since similar effects are found when ion-exchange resins are shaken with solutions containing different Ca/Sr ratios. For example, 0.05 g. samples of Zeo-karb 225 sulphonated polystyrene resin in the H-form were shaken for 1 hour with 100 ml. portions of solutions containing 12 mM (Ca+Sr), the strontium being labelled with Sr^{87} . The strontium uptake was found by counting the Sr^{87} and correcting for decay, and the calcium uptake by dry ashing the resin and determining calcium in the ash. The results shown in Table IV indicate that the resin discriminates for strontium at low levels but to a much less marked extent than tomato roots.

The results with resin and roots are not strictly comparable, since the resin took up 1.5 per cent. of the calcium supplied as against only 0.03 per cent. taken up by the roots. In addition the resin was shaken with calcium nitrate only whereas the roots were taking up ions from a full culture solution. However, we conclude that the discrimination for the small quantities of strontium shown by roots is probably a physico-chemical effect.

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Induction Phenomena of Photosynthetic Algae at Low Partial Pressures of Oxygen

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Received 14 October 1955

SUMMARY

The effect of a dark period at very low partial pressures of oxygen (10^{-4} – 10^{-1} mm. Hg) upon the subsequent photosynthetic production of oxygen by *Chlorella pyrenoidosa* with saturating intensities of illumination has been investigated. With the lowest partial pressures of oxygen used the time course of oxygen production separates into two phases; an initial burst followed by a subsequent rise to the final steady state. The separation of the two phases in time is greater the lower the concentration of carbon dioxide and the longer the preceding dark period. The initial burst of oxygen as distinct from the steady state production is not inhibited by 10^{-3} M. iodoacetamide. In contrast with the 'Hill' reaction, as measured after addition of quinone, the initial burst of oxygen was inhibited by *p*-chloromercuribenzoate (10^{-4} M.). Concentrations of sodium fluoride and of 2:4-DNP which had no effect on the steady state of photosynthesis shortened the half-time for attainment of the steady state so removing or obscuring the initial burst. The effect with fluoride was observed only if added at the beginning of the preceding dark period.

It is suggested that the initial oxygen burst results from reductive amination and carboxylation of keto acids present at the beginning of illumination. Iodoacetamide is assumed to inhibit steady state photosynthesis by preventing reduction of phosphoglyceric acid (PGA) to triose and hence its regeneration. It is assumed not to inhibit reductive amination or carboxylation. With long dark periods the initial burst is separated in time from the final acceleration to a steady state; this phase appearing as a decreased rate of production of oxygen may be also due to inhibition by fermentation products or to their conversion to amino acid without change in oxidation-reduction. The separation in time is minimized if fluoride or 2:4-dinitro-phenol is added at the beginning of the dark period; both probably prevent accumulation of pyruvic acid during fermentation limiting the initial reactions to the reduction of PGA.

INTRODUCTION

THE rate of photosynthesis during the first minutes of illumination following a dark period is generally lower than that in the subsequent steady state; this period is referred to as the induction phase. The early work of Willstätter and Stoll (1918) and later work by Warburg (1951) showed that the induction phase measured in terms of the consumption of carbon dioxide is much pro-

¹ The Hersch galvanic cell was kindly supplied by the Mond Nickel Co. Ltd., Birmingham. The author is also grateful to Dr. P. Hersch of that company for advice concerning the arrangement of the flow line.

longed if, during the preceding dark period, the partial pressure of oxygen is maintained low. A similar effect on the production of oxygen has been found by Gaffron (1935), Noack, Pirson, and Michels (1939), Franck, Pringsheim, and Lad (1945), Blinks and Scow (1938), and Hill and Whittingham (1953).

Most of these investigators used manometric methods. With this technique it is difficult to maintain a low partial pressure of oxygen in photosynthetic cell suspensions during illumination; furthermore the precise partial pressure is not known. Hill and Whittingham (1953) used a spectroscopic method with haemoglobin as indicator so that both the rate of production of oxygen and the partial pressure were determined simultaneously. This method is very sensitive within the range 2–30 mm. Hg. They found the induction phase was prolonged if the partial pressure was less than 2 mm. Hg at the beginning of the illumination, but even at the lowest partial pressures used the rate of oxygen production showed a continuous increase up to the steady state. Franck, Pringsheim, and Lad (1945) used a method for determination of oxygen dependent on the quenching of phosphorescence of certain dye-stuffs. With this method measurements were confined to partial pressures of oxygen between 10^{-6} and 10^{-4} mm. Hg. At these very low partial pressures, the oxygen production from a suspension of photosynthetic algae showed an initial burst followed by a rise of rate to the steady state. Blinks and Scow (1938) had previously found a similar effect with *Ricinus* leaves and *Uva* thallus and Gaffron (1935) with algal suspensions. The present paper describes the application of a new method for the determination of oxygen at partial pressures of oxygen between 10^{-4} and 10^{-1} mm. Hg. This fills the gap in concentration range of the two earlier investigations. With partial pressures of oxygen at the beginning of illumination less than 5×10^{-3} mm. Hg the initial burst, observed by Franck, Pringsheim, and Lad at a lower partial pressure, was found.

The effect of inhibitors on the initial burst has been investigated and compared with their effect on steady state oxygen production. On the basis of the results suggestions are made as to possible biochemical mechanisms responsible for the induction phenomenon.

EXPERIMENTAL METHODS

Chlorella pyrenoidosa (Emerson strain) was grown in pure culture in the medium described by Emerson and Lewis (1939) with a gas stream of air containing 5 per cent. carbon dioxide continuously passing through the cultures at 3 litres per hour and with continuous illumination from tungsten lamps (2×100 watt plus 2×60 watt all 30 cm. distant). Growth was at 23° C. Four-day old cultures were harvested except where otherwise stated, centrifuged at 1,000 g. for 5 minutes, washed with glass distilled water, and then resuspended in glass distilled water to which potassium bicarbonate or phosphate was added. The final suspension consisted of $13.6 \mu\text{l.}$ cells in 25 ml. of liquid. Carbon dioxide was supplied as bicarbonate solution, the concen-

tration of which was calculated from the equation given by Rabinowitch (1945):

$$[\text{CO}_2] = \frac{[\text{HCO}_3^-]}{9} \approx 10^{-3} [\text{HCO}_3^-].$$

In some experiments, not described in detail, cells were suspended in acid phosphate (M. 30 KH_2PO_4) and in this case the gas stream was previously passed through bicarbonate; the concentration in the cell suspension was not known in this case.

Scenedesmus D. 3 was grown in a similar fashion but the culture medium was that used by Horowitz in Gaffron's laboratory (personal communication).

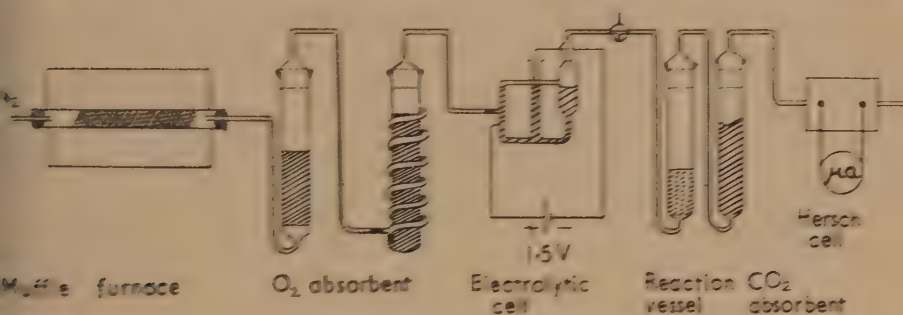


FIG. 1. Diagram of the apparatus.

Photosynthesis was determined with saturating intensities of illumination by continuous analysis of a gas stream passed through the suspension of algae at a rate of 3 to 5 litres per hour. Nitrogen free of oxygen was obtained by passing 'Dry-free' nitrogen supplied by the British Oxygen Company over heated copper (800° C.) and subsequently through a solution of anthraquinone-2-sulphonic acid (1 g.), sodium hydroxide (107 g.), and sodium hydrosulphite (75 g.) dissolved in water (1 l.). This solution is deep red and loss of efficiency as an absorbent of oxygen is shown by decolorization. A second tower circulates the liquid absorbent through a central space filled with zinc granules thus renewing the absorbent continuously (Fig. 1). The gas emerges from the second tower with approximately 1 v.p.m. (volume per million) of oxygen; the subsequent flow line must therefore be free of leaks to air. The rectangular cell which is next in line contains dilute sulphuric acid; platinum electrodes are included so that, by electrolysis, oxygen can be supplied to the gas stream. The amount of oxygen supplied per unit time can be determined from Faraday's Law of electrochemical equivalence, provided the electrodes are of small dimension. With this cell in line, calibration takes only a few minutes and in fact was found to be necessary only once per day. The gas then enters the algal suspension contained in a pyrex tube with inverted bubbler at the base. Subsequently carbon dioxide was removed by a tower containing solid absorbent and the gas then passed through the oxygen measuring galvanic cell of the type described by Hersch (1952). The current

output of the Hersch cell was determined using an Elliott recorder (200 ohms, 50 microamps f.s.d.) and later using a 'Honeywell-Brown 2-second Electronik' recorder (10 mv. f.s.d.); suitable shunts and voltage dividers provided a 10-fold increase in range. The flow rate throughout the system was recorded each minute and controlled by a mercury head.

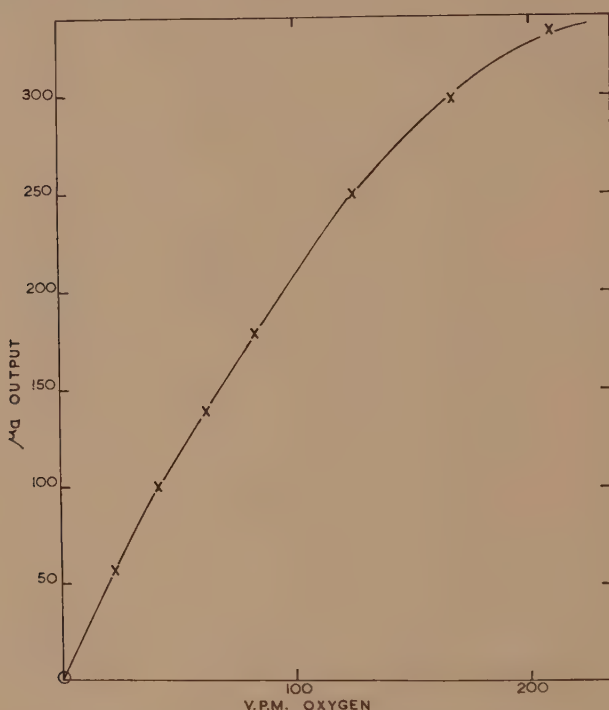


FIG. 2. Calibration curve. Oxygen was generated electrolytically. The abscissa represents coulombs of electricity which is proportional to the oxygen generated. Flow rate 5 l./hour. Resistance of circuit external to Hersch cell 200 ohms. In the subsequent figures each ordinate unit corresponds to an increase in rate of oxygen production of $10 \mu\text{l./hour}$.

Calibration showed the response up to 50 v.p.m. with a flow rate of 5 litres/hour to be sufficiently nearly linear that current output could be taken as directly proportional to oxygen concentration (Fig. 2). At 300 v.p.m. the slope of the curve of output against oxygen partial pressure was decreased to between one-half and one-third. A minimum concentration of 0.1 v.p.m. (7×10^{-5} mm. Hg) could be detected. The useful range in these experiments extended from 1 v.p.m. (7×10^{-4} mm. Hg) up to 500 v.p.m. (3.5×10^{-1} mm. Hg). This range of concentration falls between the upper limit of the phosphorescence method and the lower limit of the spectroscopic method.

In the complete system the response time of the apparatus was dependent on the flow rate. The volume between the photosynthetic reaction vessel and

the galvanic cell was approximately 200 ml.; with a flow rate of 5 l./hour no response could be detected for between 2 and 2½ minutes. The overall half-time to develop a steady state concentration of oxygen in the gas stream produced by electrolysis was of the order of 4 minutes. For production of oxygen by photosynthesis, the half-times were between 8 and 20 minutes. In the experiments reported in this paper, the phenomena under investigation have a half-time of several minutes.

The apparatus was not thermostatted; infra-red radiation was removed from the light beam and the temperature changed little during an experiment. Between experiments the temperature varied from 23° to 26° C.

The concentration of oxygen determined is, in all cases, that in the gas phase emerging from the algal suspension. This must be less than that in the liquid phase. If we suppose the bubble size to be of radius 0.05 mm. then, with a flow rate of 5 l./hour, there are approximately 3×10^6 bubbles per second. If each bubble remains in contact with the solution one-third of a second at any given time there are 10^6 bubbles in the suspension. Within the suspension as a whole, let $n = 10^6$ = the number of bubbles radius $r = 0.5 \times 10^{-2}$ cm. in which the concentration is C . Let C_2 be the concentration in the bulk of the liquid phase. Then the change of concentration with time of a bubble entering the liquid with zero concentration is given by:

$$\frac{dC}{dt} = \frac{1}{\frac{4}{3}\pi r^3} \left[K \frac{4\pi r^2}{r} (C_2 - \sigma C) \right] = \frac{3K}{r^2} (C_2 - \sigma C)$$

when K is the coefficient of diffusion of oxygen in water and σ is the solubility. Hence

$$\frac{\sigma C}{C_2} = 1 - e^{-(3K\sigma/r^2)t}.$$

The radius of the bubble, the number of bubbles, and the time of contact of bubble with the solution determine the flow rate R , as given by the equation:

$$R_a = \frac{4}{3} \frac{\pi r^3 n}{t}.$$

Given $K = 1.5 \times 10^{-5}$ cm.²/sec., $\sigma = 0.03$, $n = 10^6$, $r = 0.5 \times 10^{-2}$ cm., and $R_a = 1.39$ ml.³/sec., it follows that the emergent concentration C_1 is given by:

$$\sigma C_1 = 0.18 C_2.$$

Thus, the concentration of oxygen may be 5 to 10 times greater in the bulk of the solution than that in the emergent gas phase. This difference will be dependent on the size and number of bubbles and on the rate of flow. The estimate given merely represents an order of magnitude. Experimentally Allen (1955) found under his rather different conditions that, with a concentration of 7.6×10^{-4} mm. Hg oxygen in the gas phase, there was approximately 4×10^{-3} mm. Hg oxygen in the liquid phase.

It is also of interest to calculate the half-time of response of concentration in the gas phase for a steady rate of production of oxygen in the liquid, R ,

assumed to commence at the full rate from zero time. The change in concentration in the liquid phase with time is given by

$$\frac{dC_2}{dt} = \frac{1}{V}[R - R_a \cdot C].$$

when V is the volume of liquid present.

We assume for this calculation that the concentration in the bubbles is approximately that for when the liquid concentration is constant.

Then
$$\frac{dC_2}{dt} = \frac{1}{V}\left[R - \frac{0.18 R_a}{\sigma} \cdot C_2\right].$$

Thus
$$C_2 = \frac{R\sigma}{0.18 R_a} [1 - e^{-(0.18 R_a/\sigma V)t}].$$

i.e.
$$t_{\frac{1}{2}} = \frac{\sigma V}{0.18 R_a} = 21 \text{ secs.}$$

Thus the half-time of response of the system to an instantaneous change in rate of production in the liquid phase is 21 seconds and the asymptotic concentration is approximately one-fifth that in the liquid phase. As mentioned previously the response of the recorder will be delayed by a time interval equal to the flow time between reaction vessel and measuring system, provided that the flow is not turbulent. Comparison of the calculated half-time with the observed oxygen time course shows that even for the oxygen burst oxygen is not produced at maximal rate from the instant of illumination.

RESULTS

The half-time of the induction phase for *Chlorella* was found to be greater the lower the oxygen pressure to which the cells had been subjected in the preceding dark period. The final rate, however, was little affected by even the lowest partial pressures of oxygen used, at least with dark times of the order of one hour or less. This is in agreement with the results reported by Allen (loc. cit.).¹ He found that if dark times longer than one hour were used the steady state rate of photosynthesis was permanently diminished.

The induction half-time was found to be markedly dependent on the concentration of carbon dioxide. When the concentration of bicarbonate exceeded 2×10^{-2} M. (i.e. the carbon dioxide concentration exceeded approximately 2×10^{-4} M.) the induction half-time was relatively brief even at the lowest oxygen pressures ($t_{\frac{1}{2}} = 10$ min.) and the induction phase showed a continuous increase in rate of oxygen production up to the steady state rate. This concentration is of the same order as that required to 'saturate' photosynthesis. When the concentration was decreased the induction time was increased

¹ It is now clear that the extrapolation from a requirement for an oxygen pressure of 2 mm. Hg for rapid recovery from an anaerobic period to a similar requirement for steady state photosynthesis as implied by Hill and Whittingham (1953) is incorrect; if there is any requirement for oxygen in the steady state it is satisfied by a pressure less than 10^{-3} mm. Hg.

($t_{\frac{1}{2}} = 20$ min.) but an initial oxygen burst ($t_{\frac{1}{2}}$ for burst = 3-4 min.) was observed provided the oxygen pressure was less than 10^{-2} mm. Hg. In the experiments of Franck, Pringsheim, and Lad (loc. cit.) the gas stream passing through the culture contained 2 per cent. carbon dioxide (4.5×10^{-4} M.) but, in the liquid phase containing the cells, it will have been less than this by a factor (dependent on the conditions of experiment as discussed previously)

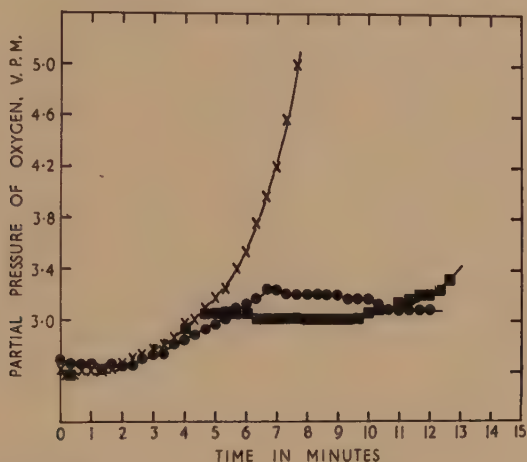


FIG. 3. Time course of oxygen production for *Chlorella*. Cells suspended in glass distilled water, ■ with no added carbon dioxide, X with 0.02 M. KHCO_3 , ● with 0.02 M. KHCO_3 plus 0.0074 M. iodoacetamide. Preceding dark time 75 min. 4-day-old cells grown in medium with urea as N. source.

which may have been as great as tenfold. Allen (loc. cit.) used the same concentration of carbon dioxide as did Franck, Pringsheim, and Lad but with more efficient stirring; consequently there would be a higher relative concentration in solution and in agreement with our results Allen found no oxygen burst, whereas Franck, Pringsheim, and Lad did. Fig. 3 illustrates the time course for oxygen production in *Chlorella* with a concentration of carbon dioxide of 2.0×10^{-4} M. together with that for cells in glass distilled water to which no carbon dioxide was added. At the higher concentration no burst can be observed; with no added carbon dioxide but with that present only from fermentation the initial production of oxygen is clearly shown.

To determine whether the initial burst was due to a physical effect which only became apparent when photosynthesis was minimized a control experiment was made with dead cells. When *Chlorella* killed by heating to 50°C . for half an hour was illuminated no oxygen production was observed, demonstrating that the initial burst was physiological.

It is not easy to separate the burst from the subsequent development of Photosynthesis at higher concentrations of carbon dioxide. It can be quantitatively determined only when the induction half-time for the development of

steady state photosynthesis is sufficiently long so that the time course separates into two phases. This is the case, for example, with no added carbon dioxide. It can then be shown that the initial burst has the same magnitude for cells suspended in M./30 potassium dihydrogen phosphate as for cells in distilled water. In acid solution little of the carbon dioxide produced in fermentation will be retained in solution; further, even when all carbon dioxide was removed from the gas stream entering the cell suspension by passing it through 5 N. potassium hydroxide, the same oxygen burst was observed. This suggests that the oxygen burst is not dependent on the presence of carbon dioxide. With high concentrations of carbon dioxide the burst could be determined only in presence of an inhibitor of photosynthesis. For this purpose iodoacetamide was used. Addition of 7.4×10^{-3} M. iodoacetamide to *Chlorella* almost completely inhibited steady state photosynthesis; but as shown in Fig. 3 it did not inhibit an oxygen burst which was comparable in amount to that observed with the same cells in absence of inhibitor with no added carbon dioxide. If it is assumed that iodoacetamide does not inhibit the reactions responsible for the burst we can conclude that the burst size is independent of concentration of carbon dioxide. The maximum bursts observed were between 1 and 2 per cent. of the cell volume, i.e. between 4.5 and 9×10^{-4} moles oxygen/l. cells.

Under the same conditions the effect of iodoacetamide on oxygen production by *Chlorella* in light after addition of quinone was determined (Hill reaction). This reaction, like the oxygen burst, was found not to be inhibited. Higher concentrations of iodoacetamide (1.6×10^{-2} M.) were found to inhibit the oxygen burst almost completely, whereas Wessels (1954) showed previously that the Hill reaction was not inhibited even by high concentrations of iodoacetamide. Thus iodoacetamide serves to distinguish the oxygen burst from steady state photosynthesis and probably also from the Hill reaction. The latter distinction was made more clear by the use of *p*-chloromercuribenzoate. As shown by Arnon, Allen, and Whatley (1954) and Wessels and Havinga (1953) this compound at concentrations of 10^{-4} M. does not inhibit the Hill reaction as measured with quinone. Addition of 1.2×10^{-4} M. *p*-chloromercuribenzoate to *Chlorella* prolonged the induction half-time and inhibited the oxygen burst to about one-half (Fig. 4); steady state photosynthesis was reduced to about 4 per cent. of the control. Inhibition could not be reversed by addition of cysteine. Thus the oxygen burst cannot be identified as a Hill reaction. Further it was found that the oxygen burst approached independence of light intensity at a much lower intensity than did photosynthesis or Hill reaction.

The effect of cyanide and of *o*-phenanthroline, both inhibitors of photosynthesis, on the oxygen time course was investigated. With a concentration of carbon dioxide of 2.0×10^{-4} M., addition of cyanide (10^{-4} M. KCN) prolonged the induction half-time and had only a small effect on the initial oxygen burst (Fig. 5); it reduced the steady state rate of photosynthesis to 65 per cent. of the control. Franck, Pringsheim, and Lad (loc. cit.) similarly reported that

cyanide had less effect during the induction phase than during the steady state. Higher concentrations of cyanide which almost completely inhibited photosynthesis did not markedly inhibit the initial oxygen burst. It was also shown that 1.2×10^{-3} M. *o*-phenanthroline had little effect on the initial

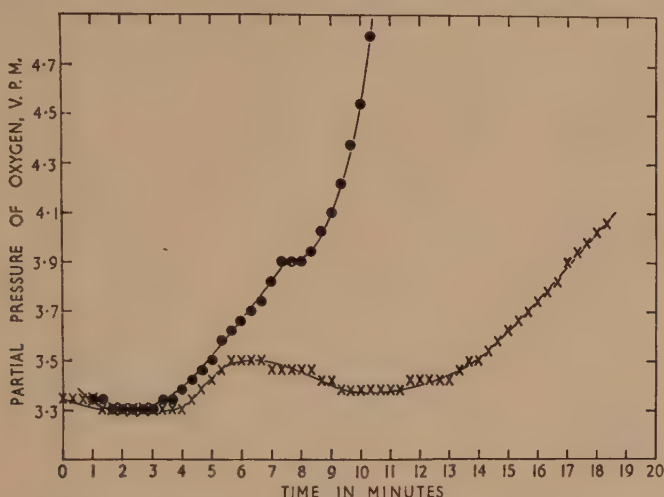


FIG. 4. Effect of *p*-chloromercuribenzoate on oxygen time course. Cells suspended in 0.01 M. KHCO_3 . 5-day-old culture; nitrate medium. 80 minutes' dark time. ● without inhibitor; X with 1.2×10^{-4} M. *p*-chloromercuribenzoate.

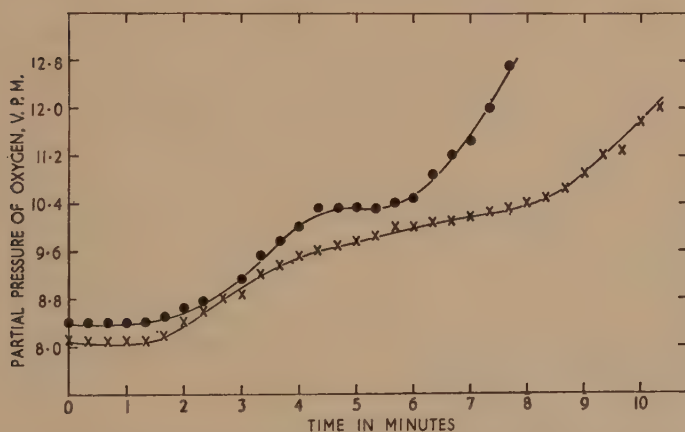


FIG. 5. Effect of potassium cyanide on oxygen time course. Cells suspended in 0.02 M. KHCO_3 . Dark time 100 min. X with 10^{-4} M. KCN.

oxygen production, whereas it completely inhibited the steady state of photosynthesis.

The effects of addition of 2:4-DNP and of sodium fluoride were similar. Both markedly shortened the induction half-time. With a concentration of

carbon dioxide 10^{-4} M., addition of 10^{-3} M. DNP almost completely removed the burst (Fig. 6); 2.7×10^{-2} M. sodium fluoride had the same effect (Fig. 7). Neither inhibitor at the concentrations used had any effect on the final steady state rate of photosynthesis. (In acid solution where much less of the DNP is ionized, the concentration of DNP used almost completely inhibited photosynthesis.) When sodium fluoride was added immediately prior to illumination

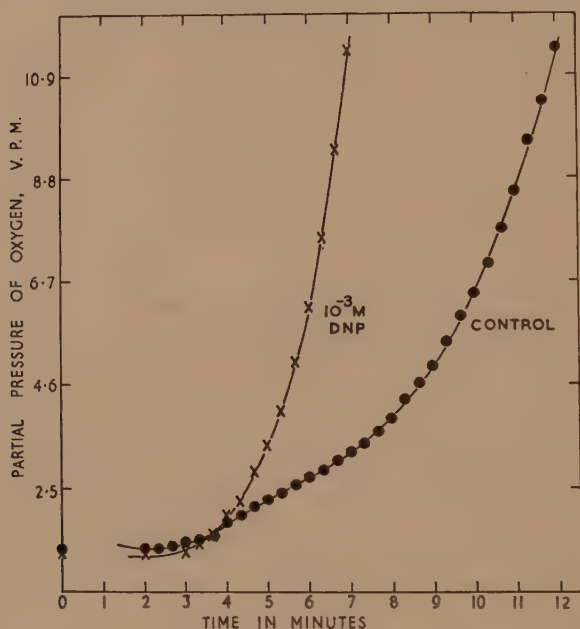


FIG. 6. Effect of 2:4-dinitrophenol. Cells suspended in 0.01 M. KHCO_3 . Dark time 45 min. X with 10^{-3} M. DNP.

and not at the beginning of the preceding dark period, there was less effect on the oxygen time course. One interpretation might be that fluoride does not rapidly penetrate to the reaction centres. Alternatively, it may be that fluoride inhibits a reaction occurring in the dark, probably the conversion of phosphoglyceric acid to phosphoenolpyruvic acid catalysed by enolase. DNP probably inhibits conversion of phosphoenolpyruvic acid to pyruvic acid. Thus both inhibitors might be expected to inhibit formation of pyruvic acid and both result in removal of the burst.

Small amounts of nitrate may be present in the measuring medium brought over from the culture medium; the initial burst might then be attributed to reduction of nitrate. To test this cells were grown with urea as nitrogen source and the effect of adding nitrate during measurements investigated. In absence of added nitrate, cells grown in urea behaved similarly to cells grown in medium containing nitrate except that a still lower concentration of carbon dioxide was necessary to separate the initial oxygen burst. Addition of 0.1 M.

nitrate did not significantly prolong or increase the initial burst; thus it is probable that the initial burst of oxygen cannot be attributed to reduction of nitrate.

The length of dark time prior to onset of illumination had a marked effect

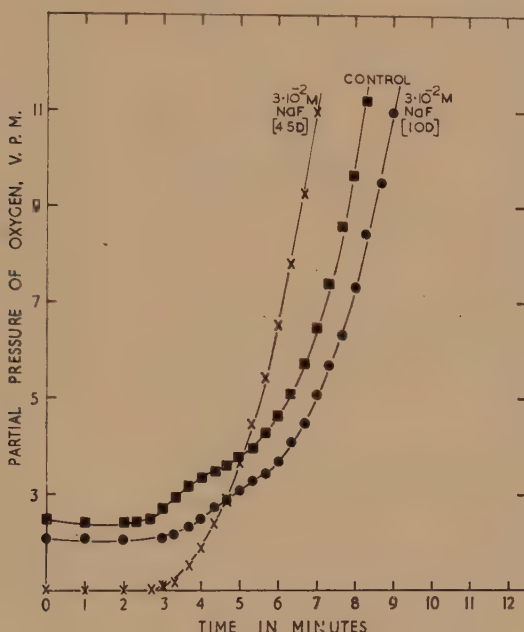


FIG. 7. Effect of sodium fluoride. Cells suspended in 0.01 M. KHCO_3 . 5-day-old culture in nitrate medium. Total dark period 45 min. ● Fluoride added 10 min. before illumination; X fluoride added at beginning of dark period.

on the time course for oxygen production (Fig. 8). The longer the dark time the greater was the separation in time between the oxygen burst and the subsequent rise to a steady state. The initial burst was itself prolonged in time. These observations suggest that the separation in time may be due to progressive accumulation of fermentation products which inhibit oxygen production immediately after the beginning of the burst.

Gaffron (1935) has previously shown, and Whittingham (unpublished) confirmed, that the half-time for induction in *Scenedesmus* D3 is not appreciably prolonged by a dark period at a low partial pressure of oxygen when the cells are suspended in alkaline solution; in *Chlorella*, a prolonged induction follows such dark treatment whether the cells are in acid or alkaline medium. When observations were made with *Scenedesmus* in the apparatus described (alkaline medium) even with no added carbon dioxide the induction half-time was shorter than with *Chlorella*. No initial burst of oxygen was separated from the subsequent steady state of photosynthesis. When photosynthesis was completely inhibited by addition of 7.5×10^{-3} M. iodacetamide, an initial oxygen

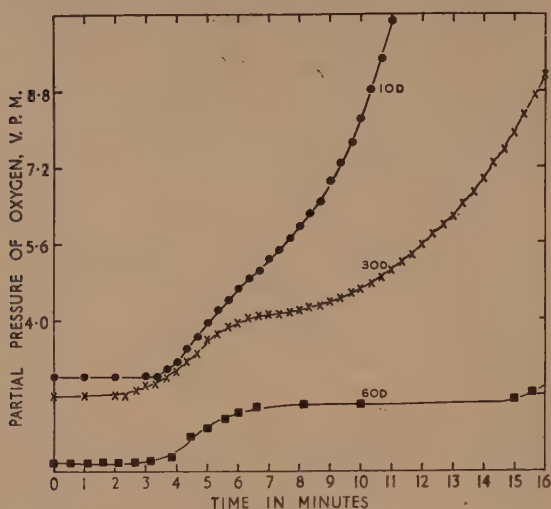


FIG. 8. Effect of length of dark time on oxygen time course. Cells suspended in 0.01 M. KHCO_3 . 5-day-old culture; nitrate medium for growth.

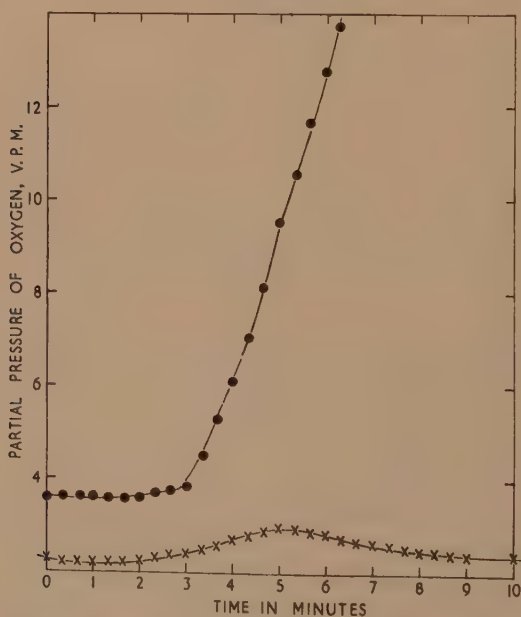


FIG. 9. Oxygen time course for *Scenedesmus* strain D3. 6-day-old culture. No added carbon dioxide. 45 minutes dark time. ● without and X with 0.0075 M. iodacetamide.

production in the light was observed similar to that found in *Chlorella* (Fig. 9). When *Scenedesmus* was suspended in acid phosphate (M./30 KH_2PO_4) with no added carbon dioxide an initial production of oxygen similar to that

in *Chlorella* was observed. Thus, *Scenedesmus* shows the same phenomena as *Chlorella* but it may remain obscured by the relatively shorter induction period in alkaline solution.

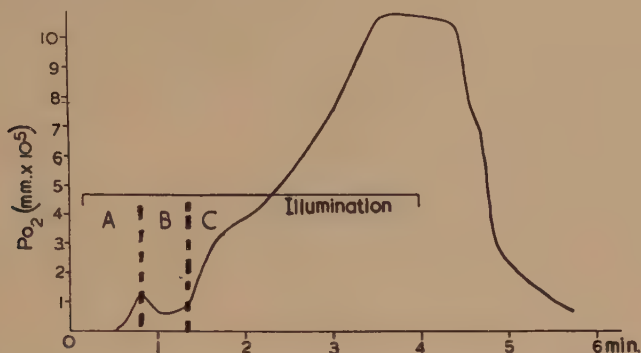


FIG. 10. The time course of oxygen for *Chlorella*. From the paper of Franck, Pringsheim, and Lad (1945).

DISCUSSION

Franck, Pringsheim, and Lad (1945) have given a detailed interpretation of the photosynthetic oxygen time course following an anaerobic dark period. They proposed that during the induction phase the normal photosynthetic mechanism operated but subject to the limitation that an enzyme catalysing the liberation of oxygen (catalyst *C*) is partially inactivated. According to their interpretation the oxygen time course may be divided into three phases (Fig. 10). During the first phase (*A*), the initial burst of oxygen, photosynthetic production of oxygen continues normally until the active catalyst *C* is 'saturated'. Precursors of oxygen should then begin to accumulate but in fact this is prevented by their reaction with accumulated fermentation products. The products of this reaction collect on the chlorophyll surface, limiting photosynthesis still further (phase *B*). This inhibitory layer is only slowly removed during phase *C* when catalyst *C* is also progressively activated and the full photosynthetic activity developed. With extreme carbon dioxide limitation there is present at the onset of illumination a reservoir or 'pool' of carbon dioxide intermediate which can be reduced in light; how far the oxygen burst is stoichiometrically equivalent to this pool size depends on the amount of active catalyst which in turn determines the amount of oxygen liberated from precursor. Further evidence in support of this interpretation was obtained from a study of the relationship of rate of oxygen production to light intensity under anaerobic conditions. Later the time course for fluorescence of *Chlorella* was found and shown to be consistent with this proposed mechanism (Shiau and Franck, 1947).

At the time of the above studies little was known of the biochemical mechanism of photosynthesis. Bassham *et al.* (1954) have now shown that certain photosynthetic intermediates are closely related to those concerned

in dark metabolism, e.g. PGA (phosphoglyceric acid). During steady state photosynthesis this substance is formed by carboxylation of ribulosediphosphate (RDP) and after reduction to triose can again give rise to further RDP as shown in Diagram I. It is not improbable that during the induction phase

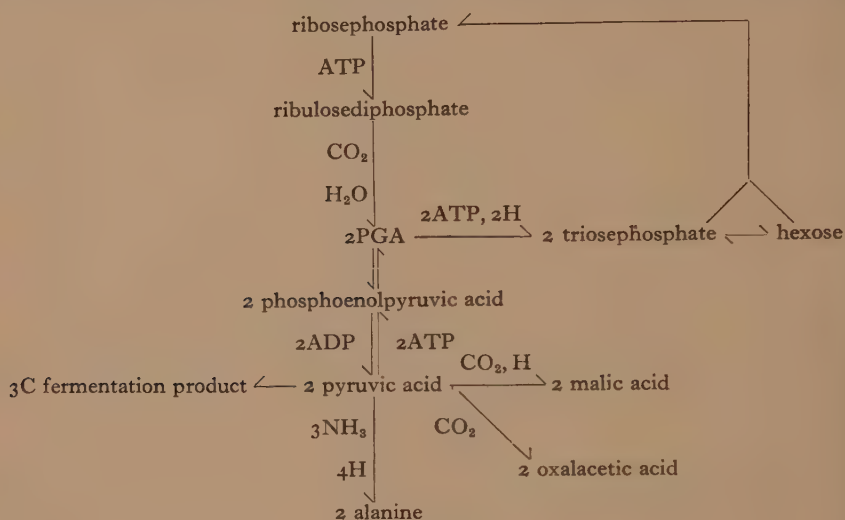


DIAGRAM I.

when this mechanism cannot be operating fully the RDP and PGA present may participate in other reaction pathways (compare Franck (1953)). Some evidence in support of this view was obtained by Krall and Burris (1954), who showed that barley after 30 sec. exposure to nitrogen and then 39 sec. exposure to light in presence of $C^{14}O_2$ showed label detectable in malic acid alone. The malic acid probably resulted from reductive carboxylation of pyruvate, which could have been derived from PGA. In other experiments Newburgh and Burris (1954), using $C^{14}O_2$, showed that in presence of iodoacetamide large amounts of radioactivity appeared in alanine. The specific activity of the alanine was greater than that of the PGA present and they suggested the existence of a second carboxylation reaction (in addition to carboxylation of ribulosephosphate) leading to fixation in pyruvic acid and hence alanine but not via PGA. Kornberg, Quayle, and Calvin (1955) have investigated the effect of various inhibitors on the carboxylation reactions exhibited by cell-free preparations from *Chlorella*. Such preparations show two types of carboxylation when supplied with ribulosediphosphate and carbon dioxide; firstly the fixation of carbon dioxide with RDP to form PGA and secondly fixation with pyruvate to form malate and oxalacetate and hence aspartate. The former reaction was not inhibited by 10^{-2} to 10^{-3} M. iodoacetate, by 10^{-2} to 10^{-3} M. fluoride, by 10^{-3} M. 2:4-DNP or by weak concentrations of cyanide less than 10^{-4} M. In the presence of iodoacetate there was increased labelling by the second carboxylation reaction probably because loss by oxidation of pyruvate

via the Krebs cycle was prevented. In the presence of fluoride on the other hand the first pathway was favoured presumably since enolase was inhibited and the formation of phosphoenolpyruvic acid and hence pyruvic acid from PGA prevented; the same result was observed after addition of DNP, in which case phosphoenolpyruvic acid tended to accumulate. Very high concentrations of cyanide (10^{-2} M.) inhibited the carboxylation of ribulosediphosphate but did not inhibit fixation into malic and aspartic acids. This might have been expected if pyruvic acid were the intermediate since this should have combined with cyanide to form cyanohydrin. Hence the carboxylation of phosphoenolpyruvic acid to give oxalacetate as shown by Bandurski and Greiner (1953) must be considered as a further possible carboxylation reaction. *p*-chloromercuribenzoate (10^{-3} M.) was found to be a strong inhibitor of all carboxylation.

These biochemical facts can be used to interpret the physiological observations reported here. Only oxygen production was measured and it is assumed that this is derived from splitting of water with the concomitant formation of reduced coenzyme. The reduced coenzyme may be utilized for the reductive amination of keto-acids to amino-acids and reductive carboxylation of pyruvic acid to malic or lactic acids. We suppose that after a dark anaerobic period there is accumulated PGA or pyruvic acid; upon illumination and the probable production of reduced coenzyme, reductive amination or carboxylation will occur. The long induction phase may be attributed to a slow development of the normal autocatalytic photosynthetic cycle in which PGA is converted to triosephosphate thence to ribulosediphosphate and further PGA. The experiments with iodoacetamide reported here show that the initial oxygen burst is little affected whilst steady state photosynthesis is completely inhibited. The inhibition is presumably due to prevention of the reduction of PGA to triosephosphate; the burst is not inhibited since pyruvic acid is still able to form alanine and hence aspartic acid by transamination. The oxygen burst was inhibited by *p*-chloromercuribenzoate which also resulted in a longer induction phase; the latter is consistent with the observation of Kornberg *et al.* that carboxylation of ribulosediphosphate is inhibited by this substance. When fluoride was added prior to the dark period the induction phase was shortened and no initial burst could be distinguished. Fluoride presumably inhibited enolase, preventing the formation of pyruvic acid and hence increasing the amount of phosphoenolpyruvic and phosphoglyceric acids. Then from the beginning of illumination the coenzyme will reduce PGA to triosephosphate. The induction phase appears then to be shorter the greater the amount of PGA present at the onset of illumination and to be longer the greater the amount of pyruvic acid. This is consistent with the view that the reduction of PGA to triose is the only reduction leading to autocatalysis. Apparently pyruvic acid competes more favourably for reduced coenzyme formed; this may be due to an additional requirement for reduction of PGA namely ATP. The latter requirement might also be related to the difference in light intensity for 'saturation' of the initial burst compared with steady state photosynthesis.

Our experiments show little influence of carbon dioxide pressure on the magnitude of the initial burst provided we accept as a measure of burst size at high concentrations that measured in presence of iodoacetamide. This observation favours a mechanism of conversion of pyruvic acid to alanine and hence possibly aspartate without prior formation of pyruvic acid via PGA from ribulosediphosphate. The higher the concentration of carbon dioxide the briefer was the induction phase, indicating that the autocatalytic cycle of ribulosediphosphate \rightarrow PGA \rightarrow triose \rightarrow ribulosediphosphate is accelerated by increasing the concentration of carbon dioxide.

Scenedesmus was shown to differ from *Chlorella*, when in alkaline suspension, in that it showed a very brief induction phase with no demonstrable initial burst. The burst could, however, be demonstrated either by addition of iodoacetamide or by suspending the cells in acid solution. By contrast *Chlorella* shows no difference in induction time course with change in pH. Allen (1955) has previously shown that the final steady state rate of photosynthesis in *Scenedesmus* in alkaline solution was not affected even after several hours anaerobiosis. Allen pointed out that change in pH frequently results in change in metabolic products of fermentation; he suggested that such an effect might account for the observed difference in induction behaviour of *Scenedesmus* in acid and alkaline solution. The mechanism discussed here would suggest that fermentation in *Scenedesmus* in alkaline solution does not result in accumulation of pyruvic acid but rather of PGA or phosphoenolpyruvic acid.

One physiological observation remains which is not accounted for by the proposed biochemical mechanism, namely the effect of increasing length of dark time. The time courses shown in Fig. 5 are not explicable simply on the basis of progressive accumulation of a single fermentation product with time. There may be additional fermentation products which ultimately result in irreversible inhibition of photosynthesis with dark periods of several hours duration at low oxygen pressures.

A further consequence of the existence of the alternative pathway for reduction of PGA is that in the initial moments of illumination the energy requirement will not be that for carbohydrate formation. Table I shows a reaction mechanism in which, provided ribosephosphate is present, one molecule of carbon dioxide could be taken up and one molecule of oxygen evolved with an energy requirement of some 70 Kg-cals. mole. In addition 1 mole of ATP would accumulate for each mole of carbon dioxide fixed. Such a reaction could be effected by 2 mole-quanta of red light (80 Kg-cals.), i.e. half the minimum possible requirement for photosynthesis.

The oxygen burst discussed in this paper is not related to the carbon dioxide burst described by Emerson and Lewis (1941). Emerson and Lewis and subsequently Brown and Whittingham (1955) both showed that the carbon dioxide burst did not occur when the preceding dark period is anaerobic. Blinks and Scow (loc. cit.) also pointed out that, in contrast with the carbon dioxide burst in light and its corresponding 'gulp' in dark, there was no uptake of oxygen in the dark corresponding to the oxygen burst in the light.

TABLE I
Free energy data

Data are from Burton and Krebs (1953) and Bassham *et al.* (1954). The free energy under standard conditions ΔF_1^0 is given. That for pH 7, 0.2 atmos. O_2 , 0.01 atmos. CO_2 , and 0.01 M. for other reactants, ΔF_1 is given in the last column.

Mechanism 1		ΔF_1^0 K cal.	ΔF_1 K cal.
1. ribose $PO_4 + ATP$	= ribosediphosphate + + ADP	?	?
2. ribulosediphosphate + $CO_2 + H_2O$	= 2PGA + 2H ⁺	-8	-7
3. 2 glycerate $3P^{3-}$	= 2 enolpyruvate $2P^{3-} +$ + 2H ₂ O	+0.8	+0.8
4. 2 enolpyruvate $2P^{3-} + 2ADP + 2H^+$	= 2 pyruvate ⁻ + 2ATP ⁴⁻	-29	-10
5. 2 pyruvate ⁻ + 2DPNH + 2H ⁺ + 2NH ₄ ⁺	= 2 alanine ⁺⁻ + 2DPN ⁺ + + 2H ₂ O	+87.1	+91.6
6. 2DPN ⁺ + 2H ₂ O	= 2DPNH + 2H ⁺ + O ₂		
ribose- $PO_4 + CO_2 + 2NH_4 + ADP$		+50.8	+75.4
		+H ₂ O	

ACKNOWLEDGEMENTS

The author is grateful to the Agricultural Research Council for financial assistance.

He also wishes to thank Prof. G. E. Briggs, F.R.S., for help in preparation of the manuscript.

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Some Aspects of Phosphate Nutrition in the Root System of Broad Bean (*Vicia faba*)

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Received 30 May 1955

SUMMARY

An attempt was made, using radiophosphate, to trace the movement of phosphate in the root systems of *Vicia faba* during the period 0 to 2 hours after commencement of uptake. The results show that phosphate inserted at an isolated point is first distributed about the root systems of turgid plants and tends to accumulate temporarily in the lateral roots before large-scale movement up the plant axis begins. This increased concentration in the laterals, which is not thought to represent metabolic accumulation, is at a maximum at 75 minutes in turgid plants but in wilted plants it terminates more rapidly. It is affected by potassium cyanide solutions.

INTRODUCTION

DURING an investigation into the movement of radiophosphate about the root system of entire plants of broad bean (*Vicia faba*), it was desired to trace the path of travel of phosphate after short uptake periods. Methods involving total immersion of the root systems were unsuitable owing to the ready adsorption of radiophosphate and the virtual impossibility of removing such adsorbed material satisfactorily (Arnott, 1954). The following method, resembling that of Moreland (1950), was therefore used throughout the experiments described below.

EXPERIMENTAL

The broad-bean plants were grown as follows: seeds of *Vicia faba* var. Barr's Monstrous Longpod were germinated in 500 ml. tall form beakers lined with folded paper towels. Tap water was placed in the bottom of the beakers to a depth of about 3 cm., so that the towels became thoroughly damp. After 3 days the tap water was replaced with culture solution (Rabideau and Burr, 1945) and this was subsequently changed every 3 to 4 days. The plants were grown in the college greenhouse and were not seriously troubled by fungal contamination.

Prior to use in experiments the plants were transferred to culture solution less phosphate for 24 hours.

Concentrations of active phosphate were determined by activity counts carried out using a mica end window Geiger-Müller tube mounted in a lead castle and connected to a power pack and scaler.

Radiophosphorus was obtained from the Atomic Energy Research Establishment, Harwell, as carrier-free orthophosphoric acid and was diluted to

10 ml. with 10^{-4} M. potassium dihydrogen phosphate solution and the pH adjusted to 5 (universal indicator) using 0.002 N. caustic soda solution. 2 ml. portions of the phosphate solution were then pipetted into hard-glass ignition tubes, each of which was fitted with a cork bored with a 5 mm. diameter hole. Experimental plants were treated with active phosphate solution by passing a selected pair of lateral roots through the hole in the cork, so that the rootlets dipped into the active solution for about 25 mm. of their length. The hole in the cork through which the lateral roots passed, and the joint between the cork and the ignition tube, were then sealed by a copious application of vaseline; the sealing was carried out with the root system and attached tube inside a 500 ml. tall form beaker, into which tap water (or other solution, as detailed later) was then gently poured until the root system was completely covered. It was necessary to take great care with the application of the seal and the introduction of the water around the root system. This procedure was practised until it could be executed within 30 to 60 seconds; if a greater time was required the plant was discarded and the experiment repeated on a fresh specimen. Once this was achieved, however, frequent observation of the air bubble inside the ignition tube, and occasional counts on samples of the water surrounding the root system, made it clear that no leakage occurred.

At the end of the experimental period the laterals in the active solution were excised at their junction with the main root, and the tube containing them and the active phosphate solution was lifted out of the beaker. The rest of the plant was blotted with a paper handkerchief and cut up into the portions required for analysis. Clean scalpels were used for the separation of these portions to minimize the risk of transferring active solution from one to the other on the blades. The portions were then cut into small pieces, dried in prepared glazed porcelain crucibles (of uniform size with flat bottoms 15 mm. internal diameter) at 120° C. for 2 hours, with occasional stirring to expose all surfaces as much as possible, and then crushed. Prior to use the crucibles were washed in hot concentrated nitric acid, then in water, dried at 120° C. for 2 hours, weighed after cooling in a desiccator over calcium chloride, and the background count determined by placing each crucible at a known fixed distance from the Geiger counter window and counting for 10 minutes. Roughly 30 mg. portions of crushed, dried tissue were counted for 10 to 30 minutes, and the background count subtracted. The crucibles and contents were then returned to the oven, dried again at 120° C. for 10 minutes, cooled in the desiccator, and weighed accurately. The net counts were then corrected to exactly 30 mg. The preparation of the crucibles was repeated before each experiment.

30 mg. was chosen as a convenient sample size since a layer of dried, ground xylem (the least dense tissue investigated here) of this weight formed a thin continuous layer over the bottom of the crucible, and more dense tissues formed even thinner layers. Self-absorption therefore did not constitute a problem. The magnitude of the counts was checked by agitating and reflat-tening several of the samples and found to be reproducible within 1 per cent.

Counts were recorded as read from the automatic register, and after correcting to equal weights were also, for comparative purposes, corrected for decay by reading back on a large-scale semi-logarithmic decay curve; the date chosen was that on which the original solution had an activity of $500\mu\text{C}$. All experiments were carried out over the temperature range 18 to 21°C .

Each experiment was designed to allow comparison of concentrations of active phosphate in two portions of the root system, and consisted of a number of replicates. In each replicate the concentrations found in one pair of samples were totalled, and one of the component concentrations expressed as a percentage of this total. Employing the percentage obtained for this component in each replication, a mean percentage for the whole experiment was calculated (cf. Snedecor (1937), p. 28). If the two components contributed equally to the total there would be no significant difference between this mean and a hypothetical mean of 50 per cent.; on the other hand if one contributed significantly more than the other, calculation of t by the method of Davies (1954) would make this apparent. In the summary of results the number of replicates and the value of t are given for each experiment.

Summary of results. A series of experiments was carried out with fully turgid plants of shoot length 15 to 20 cm., in which active phosphate was introduced into a pair of laterals about half-way along the main root. Uptake times varied from 10 to 90 minutes (Wilkinson and Lindsey, 1953); the root systems were immersed in tap water. It was found that for uptake periods of 75 minutes and less a tendency existed for P^{32} to move to the lateral roots, the maximum being reached at 75 minutes when the laterals contained a much greater concentration than the main root. At 90 minutes, as shown in the table, the tendency was less apparent. Counts taken on material from the shoot apices were negligible, so that the temporary accumulation in the lateral roots was of direct occurrence and not due to redistribution from the stem.

The results for individual plants receiving the same treatment are very variable, but attention has been drawn to this phenomenon by Scott Russell and Martin (1954).

The temporary build-up of concentration of P^{32} in the laterals, reaching a maximum at 75 minutes, is not thought to represent metabolic accumulation, but to be a stage in the phosphate circulation of the plant. It may explain the time-lag noted by Biddulph (1939) in phosphate-deficient bean plants between commencement of uptake and 'excretion' of phosphorus into the xylem, and the interval of 1 to 2 hours which Broyer and Hoagland (1943) observed between administration of radiosodium to barley plants and the steady movement of active ions into the shoot.

Although in the experiments described above phosphate was only available to the selected pair of laterals, the same result occurred at 75 minutes when the rest of the root system was immersed in an equimolar solution of potassium dihydrogen phosphate without active ion, instead of tap water (5 replicates; t for comparison of concentrations in lateral and main roots on a percentage basis, 10.54 for 4 degrees of freedom).

Quantities of P³² in roots expressed as counts/minute/30 mg.

Uptake time (min.)	Conc. in laterals above point of entry. (Net counts per min.).	Conc. in main root above point of entry (Net counts per min.).	Amount in laterals as per cent. of total.	Conc. in laterals below point of entry. (Net counts per min.).	Conc. in main root below point of entry. (Net counts per min.).	Amount in laterals as per cent. of total.
60	116	182	39	118	72	62
	30	64	32	32	82	28
	167	47	78	446	71	86
	237	667	26	175	162	52
	268	295	40	1,825	1,525	54
	101	61	62	101	50	67
	74	72	51	161	0	100
	72	41	64	95	115	45
	94	32	75	31	19	62
	1,680	134	93	238	148	62
	1,165	137	89	2,650	263	91
	1,355	320	81	472	176	73
			—			—
	Means		71			70
		<i>t</i> 2.875			<i>t</i> 3.326	
75	137	56	71	595	55	92
	210	48	81	119	30	80
	1,130	141	89	147	76	66
	2,360	425	85	169	84	67
	2,400	545	81	550	233	70
	221	185	54	233	193	55
	1,700	410	81	845	315	73
	840	36	96	456	235	66
	658	138	83	2,130	139	93
	452	96	82	258	71	78
			—			—
	Means		83			79
		<i>t</i> 8.989			<i>t</i> 7.00	
90	36	30	55	132	22	86
	168	287	37	133	266	33
	108	7	94	56	30	65
	33	22	60	41	33	55
	38	52	42	38	39	49
	183	77	70	235	93	72
	1,400	720	65	1,330	430	76
	575	272	68	102	60	64
	94	29	76	18	14	56
	250	62	80	52	20	72
	145	146	50	44	67	40
			—			—
	Means		63			67
		<i>t</i> 2.551			<i>t</i> 3.273	

If active phosphate administered as above enters the root system half-way along its length and reaches the laterals below the point of entry, it must travel via the axis in a direction opposite to that of the transpiration stream. A comparison of concentrations in stelar (mainly xylem) and extra-stelar tissues at 75 minutes showed a greater concentration outside the stele (22 replicates;

t on a percentage basis = 3.359 for 21 degrees of freedom). This state of affairs is transitory since at 90 minutes 10 out of 12 experiments showed the reverse.

The greater concentration outside the stele at 75 minutes might show either that the cortical parenchyma provided the easier route for downward travel, or that any active phosphate there was relatively immobile whilst that in the xylem moved away rapidly so that little was present at any given time. If downward travel is predominantly in the xylem, removal of the extra-stelar tissues and most of the phloem, taking precautions to minimize interference with the upward movement of ions (cf. Miller (1938) on stem-‘ringing’ experiments) should not altogether prevent it. Ten plants with secondarily thickened roots were therefore ‘ringed’ down to the main root xylem (which was protected from exposure by a coating of vaseline) and allowed to regain turgidity by standing in water for 2 hours. Active phosphate was then administered to two lateral roots above the ‘ring’, the rest of the root system being surrounded by water. Above the ring all ten showed a greater concentration in the laterals at 75 minutes; below the ring 8 out of 10 gave a higher concentration in the laterals than in the main root. The familiar 75-minute pattern can thus occur below the point of entry even though in this experiment movement was restricted to the xylem and apparently opposed the main transpiration stream.

In an effort to determine whether the state of turgidity affected the ‘75-minute pattern’ a series of experiments was carried out in which plants were wilted and then allowed to absorb active phosphate; the results were compared with turgid controls. The plants in each pair were as nearly identical as possible; the experimental plant had its root system wrapped in a damp paper handkerchief as a protection against desiccation, and was wilted at $20^{\circ}\text{C} \pm 1^{\circ}$ for a given time (15, 25, 35, 45, or 60 minutes) while the control was kept in a beaker of water for the same period. Active phosphate was then administered simultaneously to experimental and control plants for 75 minutes via a pair of laterals near the hypocotyl, so that all tissues examined were below this point. Plants wilted for 45 minutes showed a smaller concentration of active phosphate in the lateral roots than in the main (10 replicates; mean percentage 21, $t = 2.851$ for 9 degrees of freedom) whilst in the controls they contained the greater concentration (10 replicates; mean percentage 62, $t = 6.048$ for 9 degrees of freedom). The same tendency was apparent when wilting time was 60 minutes (3 replicates); however, when wilting time was 45 minutes and uptake time only 60 minutes the laterals contained the greater concentration (4 replicates). This seems to indicate that the temporary accumulation in the laterals takes place in the wilted plants, but terminates more rapidly. When plants were kept in water but fanned for 45 minutes before uptake the result was inconclusive; the control plants (not fanned) showed the usual excess concentration in the lateral roots after 75 minutes uptake (6 replicates; mean percentage 73, $t = 6.1$ for 5 degrees of freedom) whilst the experimental plants showed a smaller concentration in laterals than in main, but

this difference was not significant (6 replicates; mean percentage 42, $t = 1.51$ for 5 degrees of freedom).

The effect of potassium cyanide on the temporary accumulation was investigated by immersing the root system of experimental plants in solutions of varying strengths whilst uptake was proceeding. No change in the '75-minute pattern' could be observed using 1.7×10^{-4} M. and 5.0×10^{-4} M., but with a 7.5×10^{-4} M. solution 9 out of 10 plants showed a smaller concentration in the laterals than in the main root (10 replicates, mean percentage 28, $t = 2.896$ for 9 degrees of freedom). A potassium cyanide solution of the latter strength was not lethal since two control plants had their root systems immersed in it for $1\frac{1}{4}$ hours, then washed well in distilled water and returned to full culture solution; these plants lived and grew for 18 days and were then destroyed.

The reason for the temporary increase in concentration in the lateral roots at 75 minutes is not known; preliminary results of chromatographic and chemical analysis of extracts by the methods of Hanes and Isherwood (1949) and Albaum and Umbreit (1943) respectively indicate that the bulk of the active phosphorus is present there as inorganic phosphate and has not at that stage been elaborated.

ACKNOWLEDGEMENTS

Sincere thanks are due to Dr. A. J. Lindsey, F.R.I.C., Dr. C. L. Mer, D.I.C., and Dr. J. K. Spearing, F.L.S., for help and encouragement during the progress of this work, which was made possible by maintenance awards from the London County Council and the Agricultural Research Council.

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Proteolytic Enzymes in Growing Root Cells

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Received 4 October 1955

SUMMARY

The experimental material consisted of fifteen serial sections, each 1.0 mm. in length, cut from the apex towards the base of the bean root. Proteolytic enzymes were assayed at various pH levels on corresponding groups of sections. Values per section were converted to average values per cell by dividing each by the number of cells occurring in the section.

Evidence was obtained for the presence of two proteolytic enzymes or groups of enzymes with different pH optima. Maximum activity of proteolytic enzymes is shown by cells which have ceased to grow and in which the level of protein and certain other enzymes is falling. The significance of these observations in the interpretation of a mechanism of cell growth is discussed.

INTRODUCTION

THE present investigation amplifies an earlier one (Robinson and Brown, 1952) in which the development of the enzyme complement in the cells of the bean root was discussed. Changes in the enzyme level of the cell were investigated in relation to protein content. Four enzyme systems—an invertase, acid phosphatase, glycine oxidase, and a dipeptidase were studied. It was found that during cell growth the level of the enzymes studied increased to a maximum level and then decreased. The protein content showed a similar pattern and decreased at the same level of development as the enzyme systems. Factors which might lead to a decrease in protein content and enzyme level were considered. It seemed possible that the level of these quantities might be related to that of the proteolytic enzymes in the cell. Progressive development of proteolytic enzymes would result in increased capacity for protein hydrolysis. The present investigation was undertaken to test this possibility. The general technique was the same as that of the previous investigation (Robinson and Brown, 1952). Serial sections 1.0 mm. in thickness were cut from the apex backwards to a distance of 15.0 mm. Enzyme determinations were made using groups of sections. The activity per cell was calculated from the experimentally determined section values by dividing each by the number of cells contained in the section.

The optimum pH value for proteolytic enzymes in the bean root was not known and therefore a series of observations was made at different pH levels. The proteolytic enzymes were extracted quantitatively from the sections by the use of suitable extraction fluids.

In an earlier paper (Robinson and Brown, 1954) it was shown that changes in enzyme activity occur when isolated bean root sections are cultured on

2 per cent. sucrose. Under these conditions invertase activity decreases very markedly and phosphatase activity increases slightly; there is no alteration in protein content. The proteolytic enzyme activity of sections placed under similar conditions was determined.

MATERIALS AND METHODS

Experimental material. The experimental material used for the investigation was roots of broad bean (var. Aquadulce) seedlings. Serial sections 1.0 mm. in thickness were cut from the apex backwards from roots 3.0–4.0 mm. long taken from seedlings grown in moist sand under standard conditions of temperature and humidity. The techniques for culturing beans and sectioning roots were described in an earlier paper (Robinson and Brown, 1952).

Culture of isolated sections. The technique used for culture of isolated sections is described in an earlier paper (Robinson and Brown, 1954). Sections were cultured on sintered glass disks placed inside Petri dishes containing a suitable culture fluid. The cultures were incubated at 25° C. for various periods of time. After culture the sections were removed, washed, subjected to freezing treatment, and assayed for enzyme activity. In the present investigation sections were cultured on 2 per cent. sucrose and on water for periods of 12, 24, 36, and 48 hours. The enzyme activity was assayed at pH 3.0. Each experiment was repeated on three different occasions and the data given in the next section are the mean of these results.

Estimation of proteolytic enzymes. Enzyme estimations were made on sections which had been subjected to the freezing treatment used previously (Robinson and Brown, 1952). Sixty sections 1.0 mm. in thickness from each region of the root were placed in a small specimen tube containing 2.0 ml. distilled water. The tubes were placed on a tray, containing a mixture of ice and salt and placed in the ice chamber of the refrigerator for 1 hour. The tubes were allowed to thaw out in the laboratory, after which the sections were removed, washed, and placed in conical flasks each containing 2.0 ml. substrate solution. The flasks were shaken in a water-bath at 37° C. for four hours. The technique of proteolytic enzyme assay was that described by Anson (1939). Haemoglobin is the substrate and the tyrosine and tryptophane released on hydrolysis are estimated colorimetrically with Folin reagent, phosphotungsto-phosphomolybdic acid (Folin and Ciocalteu, 1927). The sections were placed in a 2 per cent. solution of haemoglobin (Armour Laboratories, U.S.A.) of suitable pH. After incubation for 4 hours at 37° C., the sections were separated from the substrate by filtration and then washed with distilled water. Washings and filtrate were placed together in a flask and 10 ml. 0.3 N. trichloroacetic acid added to precipitate the unhydrolysed haemoglobin. The precipitate was left for about an hour to flocculate and then removed by filtration through a Whatman No. 3 filter-paper. The clear filtrate was made up to 25 cc. with distilled water. The Folin reagent was prepared according to the author's instructions (Folin and Ciocalteu, 1927) and diluted with twice its volume of water before use (Anson, 1939). Replicate

estimations were made on 5.0 ml. aliquots of the filtrate; to each, 10 ml. of 0.5 N sodium hydroxide and 3.0 ml. of diluted Folin reagent was added. The intensity of the blue colour developed was estimated in a Hilger-Spekke absorptiometer using a 605 red filter. A calibration curve for the method was obtained using standard solutions of tyrosine.

Bean roots contain an active tyrosinase as is indicated by the blackening of the tissue in air. It was possible therefore that some of the tyrosine produced by the hydrolysis of haemoglobin might be consumed by the root sections. To test this possibility sections were placed in solutions of tyrosine at pH 3.0 and also at pH 7.0 and incubated for 4 hours at 37° C. At the end of the incubation period the sections were removed and the tyrosine estimated with Folin reagent. It was found that under the conditions of the experiment tyrosine was not consumed at either pH. It is considered therefore that the estimations made of the hydrolysis products of haemoglobin give an accurate representation of the extent of hydrolysis.

Enzyme estimations were made on 15 serial sections at three pH values—3.0, 5.0, and 7.0. It was found that buffer solution (potassium hydrogen phthalate+hydrochloric acid) at pH 3.0 induced slight precipitation of haemoglobin. Estimations of enzyme activity at this pH were therefore made with aqueous haemoglobin solution acidified to pH 3.0 with hydrochloric acid. A comparison of the values obtained using buffer at pH 3.0 and hydrochloric acid at pH 3.0 were approximately the same, probably due to the haemoglobin concentration being in excess of that required for enzyme saturation. Estimations at pH 5.0 and pH 7.0 were made using acetic acid+sodium acetate buffer and potassium hydrogen phthalate+sodium hydroxide buffer respectively.

In order to amplify the results obtained on effects of pH on serial sections from the intact root a second set of experiments was performed. A large number of sections from one region was assembled and groups of sixty placed in different buffer solutions. Thus the effect of pH on one section on the same occasion was obtained. The solutions used were pH 2 and 3 dilute hydrochloric acid, pH 4 and 5 sodium acetate+acetic acid, and pH 6, 7, and 8 potassium hydrogen phthalate+sodium hydroxide.

Expression of results. The experimental values obtained are expressed in terms of mg. amino-nitrogen produced per section. The number of cells in each case was determined using the cell maceration technique of Brown and Rickless (1949). The section figures were converted to values per cell by dividing each by the number of cells in the section.

Errors of estimation. As was pointed out in the earlier investigation (Robinson and Brown, 1952) the day to day variability of the experimental material is high. All the figures given in the next section therefore are the mean of four different observations made on four different occasions. The variability in the day to day observations is illustrated in Table I. Despite the variation the individual observations show the same general trend and therefore the mean figure of four observations is considered to indicate conditions in the root.

TABLE I

*Four replicate estimations of proteolytic enzyme activity at pH 3.0*Figures are mg. $\times 10^{-4}$ amino-nitrogen produced per cell in 4 hours

Section (mm.)	1	2	3	4
0-1	9.1	7.8	5.4	3.8
1-2	5.0	3.8	4.2	3.0
2-3	5.6	4.5	5.1	3.9
3-4	7.6	7.1	6.3	3.8
4-5	9.4	7.9	6.1	7.0
5-6	6.9	10.1	6.4	8.6
6-7	11.6	16.2	9.1	14.5
7-8	17.0	18.4	12.5	27.8
8-9	17.5	17.1	13.3	29.4
9-10	23.4	21.4	18.9	30.8
10-11	23.2	21.5	19.0	25.2
11-12	19.2	26.5	15.7	22.6
12-13	19.2	20.5	15.4	26.9
13-14	16.6	19.8	12.7	25.4
14-15	15.5	14.2	13.1	24.9

Extraction of enzyme. Bean root extracts contain substances which give a blue colour with Folin's reagent. When estimating proteolytic enzymes in tissue extracts it is necessary to make two preparations, one of which is used as a control and the other for experimental purposes. A Potter-Elvehjem glass homogenizer was used to prepare the extracts. Sixty sections and 1.0 ml. of extraction fluid were placed in the mortar which was surrounded by a beaker containing powdered ice. The homogenate was placed in a conical flask; 1.0 ml. extraction fluid was used to wash the homogenizer and the washings added to the contents of the flask. For each region of the root a duplicate extract was prepared; 1.0 ml. haemoglobin substrate was added to both homogenates. One was incubated for 4 hours at 37° C., unused haemoglobin precipitated and tyrosine and tryptophane estimated; in the other the haemoglobin was immediately precipitated and tyrosine and tryptophane estimated. The difference between the first and second values is a measure of the proteolytic enzymes in the homogenate.

Extractions were made with two fluids: hydrochloric acid at pH 3.0 and a buffer solution used by Sandegren and Klang (1950) for extracting barley proteinase. The buffer solution consisted of 46.8 g. sodium chloride, 39.4 g. sodium acetate, and 18.35 g. of acetic acid in 1.0 litre of distilled water. It was used at full strength and diluted by 2. The pH of the solution was 4.0. The results given in the next section are means of three observations.

RESULTS

Proteolytic enzymes in the intact root. The proteolytic enzyme activity of successive sections and the values per cell obtained when estimations are made in hydrochloric acid at pH 3.0 are shown graphically in Fig. 1. The curve for the section values (Fig. 1B) is rather complicated with two peaks at 3.0 and 10.0 mm. from the apex of the root. The values for the average cell in

each section (Fig. 1A) show a slight decrease in the first 1.0 mm. and then a steady rise to 10.0 mm. followed by a decrease to 15.0 mm. When these figures are compared with those in the earlier paper (Robinson and Brown, 1952) it will be observed that maximum activity per cell of proteolytic enzyme occurs at a point more remote from the apex than the other enzymes studied. Inver-

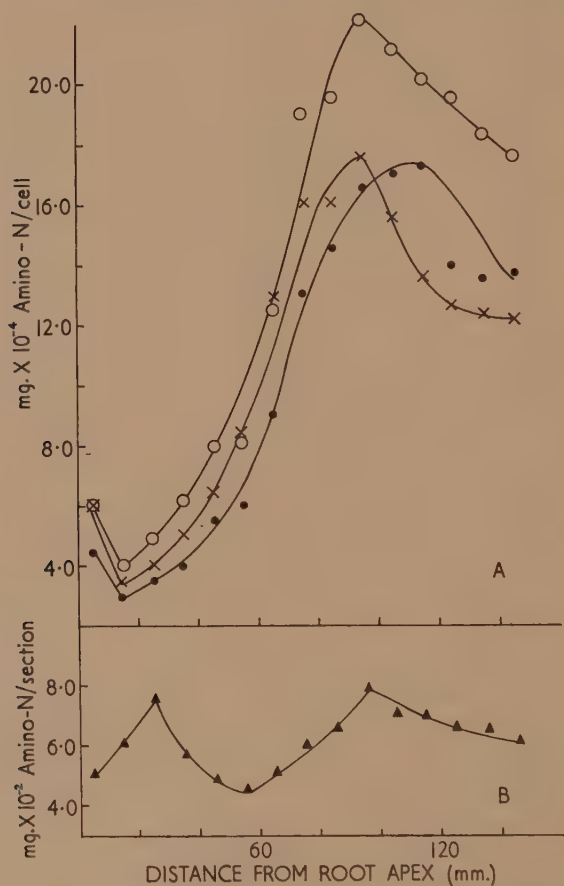


FIG. 1A. Proteolytic enzyme activity per cell at pH 3.0 (—○—), at pH 5.0 (—X—), and at pH 7.0 (—●—).

B. Proteolytic enzyme activity per section at pH 3.0.

tase, phosphatase, glycine oxidase, and dipeptidase all had maximum activity at 8.0 mm. from the apex.

The values obtained when a series of estimations was made at different pH values are also recorded. The three curves run roughly parallel to a distance of about 7.0 mm. from the apex, with highest activity at pH 3.0 and lowest at pH 7.0. In regions more remote from the apex the curves diverge. A peak value is obtained at 10.0 mm. from the apex at pH 3 and also at pH 5.0,

where the maximum is lower. At pH 7.0 the activity continues to increase to a distance of 12.0 mm. from the apex, after which it decreases. Activities at pH 5.0 are consistently lower than those at pH 3.0; at pH 7.0, however, the initial values are lower but they gradually increase to a maximum at 12.0 mm. These observations suggest that in the bean there may be two proteolytic

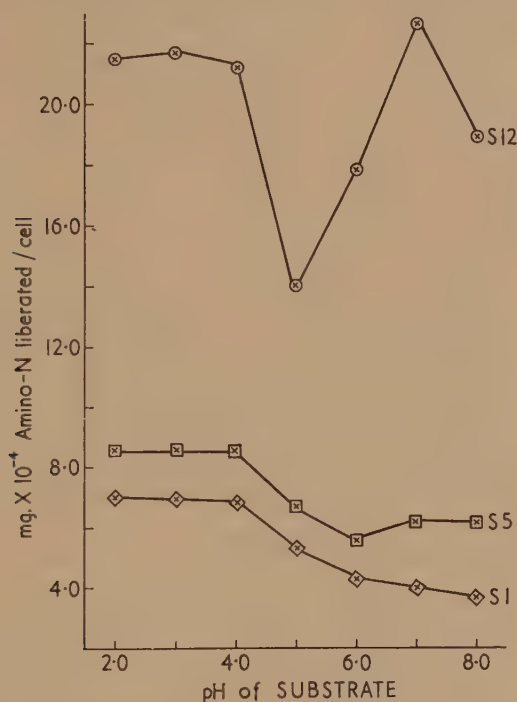


FIG. 2. Effect of pH of substrate on proteolytic enzyme activity of 3 sections: S1, 0-1.0; S5, 4.0-5.0, and S12, 11.0-12.0 mm. from the root apex.

enzymes or groups of enzymes, one with a pH optimum at 3.0, and the other at 7.0. This hypothesis was tested by taking representative groups of sections from the same region of the root and estimating enzyme activity at different pH values. The figures for three different sections—0-1.0, 4.0-5.0, and 11.0-12.0 mm. from the apex are recorded in Fig. 2. At pH 2, 3, and 4 the first section shows high activity; from pH 4 to 8 the activity gradually decreases. Activity of section 5 is high up to pH 4.0 and then decreases, showing a very slight rise at pH 7.0. Section 12 has high activity at pH 2 to 4, a fall at 5.0, and a maximum at 7.0. Thus it would appear that in the tip of the root there is only one enzyme or group of enzymes with a pH optimum at 3.0, but at some distance from the apex, probably at about 5.0 mm., a second enzyme or group of enzymes is developed with a pH optimum at 7.0.

Enzyme activity of extracts. Results obtained with extracts from sections

prepared in the manner previously indicated are shown in Table II. The values are corrected for the initial internal tyrosine and tryptophane content of the tissue.

TABLE II

Comparison of proteolytic enzyme activity in sections and extracts

(Extract values corrected for blank are means of 3 observations expressed as mg. amino-nitrogen $\times 10^{-4}$ produced per cell in 4 hours)

Region (mm.)		0-1	1-2	2-3	3-4	4-5
HCl pH 3.0	Sect.	6.4	4.7	4.9	4.5	6.0
	Ext.	2.2	2.4	2.2	1.6	1.8
Sandegren's fluid	Sect.	5.3	3.2	3.0	4.0	3.6
	Ext.	4.2	2.8	3.4	3.4	3.8
Sandegren's fluid dil. $\times 2$	Sect.	5.9	4.2	4.5	5.0	5.7
	Ext.	5.9	4.4	4.4	5.2	5.8

The activity of extracts prepared with hydrochloric acid at pH 3.0 was much lower than that of the intact sections. Extraction with Sandegren's buffer extraction fluid yielded preparations with a higher activity than with hydrochloric acid, and when used at half strength the extracts had approximately the same activity as the intact sections.

Proteolytic enzyme activity of isolated sections in culture. Data on the proteolytic enzyme activity of sections after culture on 2 per cent. sucrose and on water for various periods of time are assembled in Table III.

TABLE III

Proteolytic enzyme activity per cell (mg. amino-nitrogen $\times 10^{-4}$ per 4 hours) in sections cultured on 2 per cent. sucrose and on water

Figures at heads of vertical columns represent culture period in hours

Section No.	(a) 2 per cent. sucrose					(b) Water				
	0	12	24	36	48	0	12	24	36	48
1	7.3	6.1	8.2	6.5	7.7	6.6	7.6	5.8	7.2	4.8
2	5.7	4.4	6.8	4.6	6.5	4.4	4.6	4.2	4.1	2.9
3	5.8	4.9	6.3	5.3	6.2	5.0	5.4	4.4	5.3	3.4
4	7.5	7.8	8.7	7.3	5.8	7.1	8.2	6.6	7.8	5.3
5	8.5	11.2	7.8	10.9	7.1	8.1	10.4	8.6	7.4	5.2
6	10.4	11.1	11.3	11.8	10.6	9.4	10.7	9.9	7.8	9.1
7	13.6	13.9	16.5	14.4	15.6	12.2	13.7	13.3	12.3	9.1
8	20.3	19.9	22.8	20.1	21.4	17.0	18.3	16.6	16.6	12.5
9	21.0	20.6	22.5	20.2	23.5	17.0	18.3	16.6	14.7	12.0
10	21.7	20.9	25.5	20.3	24.5	17.7	18.3	17.7	14.8	12.2

It will be observed that after culture on 2 per cent. sucrose for periods up to 48 hours there is no alteration in the proteolytic enzyme activity of isolated sections (Table IIIa). The small variations which occur in the figures are within the limits of experimental error. After culture on water (Table IIIb) for 12 hours the proteolytic enzyme activity of all sections is the same as that of the control sections cut directly from the intact root. During 12-36 hours of culture the enzyme activity of the first four sections remains constant and

then from 36 to 48 hours it decreases slightly. In the remainder of the sections the proteolytic enzyme activity decreases during culture from 12 to 48 hours.

DISCUSSION

The results of this investigation indicate that the proteolytic enzymes may play an important part in the regulation of protein content and the level of certain other enzymes during cell growth.

Tissue homogenates have been prepared the activity of which is comparable with that of intact sections. Similar results with an acid phosphatase were obtained in an earlier investigation (Robinson and Brown, 1952). The present data confirm that enzyme estimations made on sections subjected to the freezing treatment to destroy cell membranes give a representative picture of conditions in the cell. Using tissue homogenates a study is being made of the distribution of enzymes among the various cellular components and this will be reported in a later paper.

From the data described above it would appear that there are two proteolytic enzymes or groups of proteolytic enzymes in the bean root. They can be distinguished by their different pH optima, one at pH 3.0 and the other at pH 7.0. The enzyme or group with pH optimum 3.0 is present in cells at all stages of development. This feature was also shown by the enzyme previously studied (Robinson and Brown, 1952). It would appear, however, that the enzyme or group with pH optimum 7.0 is absent from cells of the root apex (Fig. 2) and only begins to function at a distance of 4.0–5.0 mm. from the apex. The results of the earlier investigation and the data on the proteolytic enzyme or group with pH optimum 3.0 indicate that the enzymic composition of the growing cell may be altered by changes in the balance of enzymes already present. The data on the other enzyme or group of proteolytic enzymes show, however, that the composition may also be altered by the formation of new enzyme systems during growth.

Robinson and Brown (1952) showed that the activity of a dipeptidase, acid phosphatase, glycine oxidase, and invertase was maximal at 8.0 mm. from the apex of the root. The proteolytic enzyme group with pH optimum at 3.0 has maximum activity at 10.0 mm. and that with pH optimum 7.0 at 12.0 mm. The protein content of the cell showed a very similar pattern to that of enzyme activity, with a peak value at 8.0 mm. from the apex. The activity of the proteolytic enzymes continues to increase, therefore, even though the level of protein in the cell has begun to decrease. This difference between proteolytic enzymes and the others is further stressed if the relative activity per unit protein is calculated.

The results of the two earlier investigations were each related to cell growth. It was shown (Robinson and Brown, 1954) that during culture of excised fragments on 2 per cent. sucrose invertase activity decreased markedly and phosphatase activity increased. In the present investigation it was found that the activity of the proteolytic enzymes remained constant during culture

TABLE IV

Relative proteolytic enzyme activity per unit protein at pH 3, 5, and 7

(The lowest value found is taken as unity)

Section(mm)	pH 3.0	pH 5.0	pH 7.0
0-1	1.6	1.9	1.5
1-2	1.0	1.0	1.0
2-3	1.3	1.3	1.2
3-4	1.4	1.0	1.2
4-5	1.5	1.4	1.5
5-6	1.3	1.6	1.3
6-7	2.0	2.3	1.8
7-8	2.2	2.1	2.1
8-9	2.5	2.4	2.5
9-10	2.8	2.3	2.6
10-11	2.7	2.2	2.7
11-12	2.6	1.9	2.8
12-13	2.7	2.0	2.2
13-14	3.0	2.3	2.7
14-15	2.6	2.0	2.8

of excised fragments on 2 per cent. sucrose. During culture on water, however, the enzyme activity does decrease (Table III). The proteolytic enzymes differ therefore from phosphatase and invertase, the activity of which altered during culture on 2 per cent. sucrose. The protein level of the cell remains constant if 2 per cent. sucrose is supplied but decreases if water is the culture fluid (Robinson and Brown, 1954). It was concluded in the case of invertase and phosphatase that the enzyme level was independent of the protein level, as the changes occurred while the protein level was constant. It would appear, however, that the level of proteolytic enzymes is intimately bound up with that of the protein, and that decrease in enzyme only occurs under conditions where the general protein level of the cell is decreasing. In one respect, however, the results of the present investigation resemble those of the earlier one. It was noted that enzyme changes did not occur to the same extent in all fragments. There was little or no change in S₁ and S₂, relatively little in S₃ and S₄, and the most marked changes occurred in fragments S₅-S₁₀. The data of Table III *b* display the same characteristics. The protein of the proteolytic enzymes becomes more labile with increasing distance from the root apex.

Brown, Reith, and Robinson (1952) suggested that the growth of the cell may be interpreted in terms of changes in the quantities of a particular group of enzymes and of their activities as determined by factors such as substrate concentration. The quantitative level changes during development and after reaching a certain peak state decreases until a critical level is reached at which growth ceases. These ideas have been developed as the result of further experimental evidence (Brown and Robinson, 1955). It was shown earlier (Robinson and Brown, 1952) that when the level of certain enzymes decreases growth ceases, and that this decrease is determined by the protein level. The factors that govern the protein level are not known, but it may be that the proteolytic

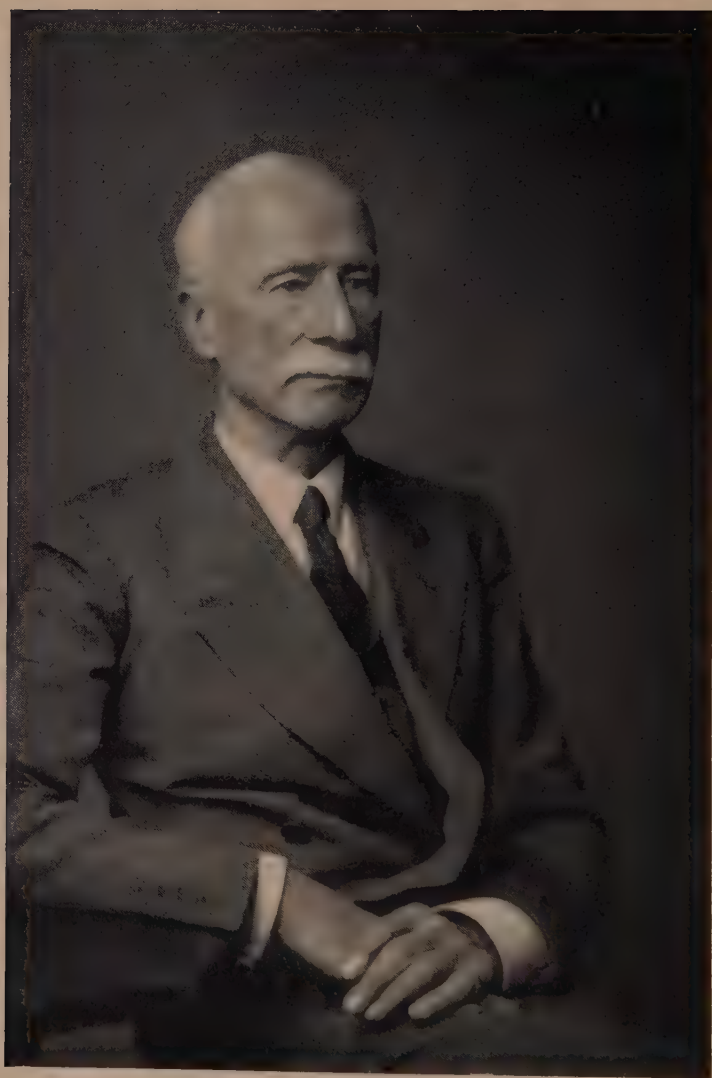
enzymes are involved in the breakdown process. As the cell grows from the meristematic to the mature state there is a gradual increase in the level of proteolytic enzymes and it is probable that the balance between factors causing synthesis and breakdown of protein gradually alters. At a certain point the two become equal and protein synthesis ceases. This occurs in the region 8.0 mm. from the tip. The proteolytic enzyme activity continues to increase and therefore breakdown exceeds synthesis and the level of protein falls in regions more remote from the tip than 8.0 mm. The decrease in protein level eventually affects the proteolytic enzymes themselves and in the region 10.0–12.0 mm. from the root apex their activity begins to fall due to the breakdown of enzyme protein. Thus the results of the present investigation suggest that the protein level of the cell is decreased by the relative increase in proteolytic enzymes and that the level of these enzymes is a factor which determines growth.

It is a pleasure to record my thanks to Dr. R. Brown for advice and criticism during this investigation and to Miss B. K. Robson and Mr. C. H. Ramsden for technical assistance.

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V. H. Blochman

Botanical Retrospect¹

V. H. BLACKMAN

A CENTENARY meeting is a time for looking backward; so when I was asked to undertake on this occasion some kind of botanical retrospect I felt, as the Society's oldest member—at least on the botanical side—that I ought not to refuse. Accordingly I suggested that I might give some slight survey of such changes during recent decades in the experimental side of botany that seemed to me important. I had in mind such innovations as the rise of new branches of plant physiology; and also the changes—which have been very great—in the methods available to the experimental botanist. The outlook must necessarily be a very individual one, and from the time available and from my competence a very imperfect one.

Members will remember that for many years few of the botanical contributions brought before the Society appeared in its journal. It is true that volume 1 of the journal, then entitled *The British Journal of Experimental Biology* (the term *British* not being dropped until volume 7 of 1930), contained four botanical papers; these, curiously enough, were all genetical in nature, though genetics was soon to disappear altogether from the journal. In subsequent volumes, contributions relating to plant studies rapidly decreased, and up to volume 28 of 1951 half the volumes had none at all. The position was of course entirely altered when, in 1950, *The Journal of Experimental Botany* was founded under the auspices of the Society.

The *botanical* papers published by the Society from 1924 to 1950 thus, in themselves, provide little material for a survey of changing studies and changing methods. Also, to one of my age the period of roughly thirty years which has elapsed since the foundation of the Society is a comparatively short one. So I would propose to go in retrospect somewhat farther back than 1932.

I may say that I did my first research work in the middle 1890's; and as a student I think I had two great advantages. In those days one was not too severely—that is not too copiously—taught, so one had time to read original papers in one's subject; then, of course, far less-extensive and far less-specialized than it is today. Secondly, the fields of related subjects were also narrower and simpler. I had the good fortune to receive, in addition to botany and zoology, some training in physics, chemistry, and human physiology. As a result I was for a good many years able to understand, at least the import, of most of the letters appearing in *Nature*. The position, I need hardly say, has for a long time now been sadly changed.

The field of research in which I first worked was cytology, and as there was no school of plant cytology in this country I went to the University of Bonn. The remoteness of those early days, sixty years ago, may perhaps be brought

¹ An address given at the 100th meeting of the Society for Experimental Biology, held at Cambridge on 13 July 1955.

home to members by an incident of those times. Professor Strasburger, the head of the department and then the most eminent cytologist of the day, came into the laboratory to announce the discovery by Röntgen of what are now known as X-rays. I can well recall the gesticulations with his fingers with which he sought to bring home to his foreign students—many of them with a very imperfect knowledge of German—the amazing fact that by means of these rays the bones of the hand could be photographed through the flesh.

At the time I was working in Bonn there was no subject of cytogenetics, in fact there was no body of knowledge which could be so described. Since the behaviour of the chromosomes could at that time in no way be linked with any mechanism of heredity, cytology seemed likely to become little more than a branch of what might be described as micro-anatomy. Thus to one primarily interested in plant processes it lost its attraction.

The chromosome basis of heredity was not to be laid until well after 1900, when the forgotten work of Mendel was brought to light; and I well remember the excitement among biologists that its rediscovery aroused. That rediscovery resulted also in the well-known and embittered controversy between the biometric school and the Mendelian school, with Raphael Weldon and William Bateson in opposite camps. Bateson, who lived near me in Wimbledon, was a master of polemics and most quick-witted in controversy. I think I should put on record one of his controversial exchanges. At a meeting of the Evolution Committee of the Royal Society, Weldon had read a paper on the size of the carapaces of a certain population of crabs. Bateson, who considered the results of no biological importance, when asked by the chairman of the Committee to comment upon Weldon's contribution did so in a single devastating sentence: 'Though all science might be measurement, all measurement was not necessarily science.' I may say that I have occasionally found it useful to bring this dictum before research students, who are sometimes inclined to believe that so long as they are measuring something they must be advancing science.

Statistics. In those early days statistics as an ally in general biological experimentation—an invaluable associate now taken for granted—was quite unknown. The theory of probability came into agriculture from astronomy, and then from agriculture into general biology; and the date of entry can be fixed as 1910-11. In 1910, at the Sheffield meeting of the British Association, a group of four papers on agricultural yields and their interpretations was read at a joint meeting of the sections of Agriculture and of Economics. These papers, modified and amplified, appeared in November 1911 as Supplement VII to the Journal of the Board of Agriculture, and thus became available to biologists generally.

In that supplement the central article was one in which elementary statistical treatment was applied to the yield-results of certain agricultural field trials. The author was T. B. Wood, then professor of agriculture at Cambridge, who acknowledged assistance on the mathematical side from F. J. M. Stratton, who later became professor of Astrophysics at Cambridge. I can well recall the

feeling I had on reading that supplement forty-four years ago; that here the biologist had acquired something entirely new, for he had been provided with a tool—hitherto wholly lacking—for testing the validity of the conclusions drawn from his experimental results. The tool provided was then no more than a method of calculating the probable error of a set of data and of determining the significance of a difference of means. It was not until eight years later that R. A. Fisher joined the staff of the Rothamsted Experimental station and applied his mathematical genius to the development of statistical methods for research workers.

The rise of new branches of biology. I have already referred to the comparatively late appearance of cytogenetics as a special branch of biology; but there were other fields of biology which were almost equally unknown, for example, that of *viruses*. It is true that Ivanovsky in 1892 had demonstrated in tobacco mosaic the existence of a filter-passing infective agent, but there was as yet no general acceptance of viruses as a special group of such agents. Also, the observation of virus particles and the knowledge of crystalline viruses lay well ahead. The related *bacteriophages* had yet to disclose themselves; enzymology also, in those days, was in an almost infantile condition compared with its stature and development at the present time. In addition, such important fields of work as *photoperiodism*, *plant auxins*, and *vernalization* had yet to be opened up. Photoperiodism dates from the work of Garner and Allard in 1920, a little before our founding date; while the demonstration and the isolation of plant auxins resulted mainly from the work of F. W. Went and that of Kögl in the late 1920's and early 1930's. Vernalization may be dated from the work of Gassner in 1918, but it was not developed on modern plant-physiological lines until the early 1930's.

Methodology. To pass more definitely to the matter of experimental methods, the most powerful tools that have been put into the hands of the experimental botanist since the first meeting of the Society in 1923 would seem to be two in number. One is the labelling of substances of biological importance with radioactive atoms; by such means the substance can be recognized in amounts far smaller than is possible by the most sensitive chemical or spectroscopic methods; also, if required, the path of the substance can be followed with ease through the body of a complex organism. Furthermore if the substance is broken up in metabolism the appearance of the radioactive atoms in new combinations can be made evident.

The second of the two techniques, which would seem to vie with the first in effectiveness and power, is *paper-partition chromatography*, by which the components of a mixture, even though closely related chemically, can be separated from one another and, by appropriate methods, identified. Each of these two techniques might be described as a revolution in chemical-analytical methods. When they are used in combination they provide the investigator with a tool of incomparable power.

To consider first the use of radioactive isotopes, known in U.S.A. as *tracer methodology*. The earliest physiological experiments with active isotopes were

made in the early 1920's by Hevesy who used the heavy elements lead-210 and bismuth-210, and followed their absorption and distribution in the bean plant and in the guinea-pig. The physiological results were not of great importance but the value of the method was demonstrated. To the biologist, however, little might have come from this new technique if the invention of particle-accelerating machines and of the reactor had not made possible the production of radioactive elements of *low molecular weight*, that is, of elements which play a part in normal physiological processes.

Some of the earlier workers struggled with the difficulties presented by carbon-11. They had to prepare and purify the labelled substance, carry out an experiment, and then determine the amount of the isotope taken up; and all this had to be done within the 10 or 15 minutes which is all that its 20 minutes of half-life allows. In spite of such obstacles to experimental work, Ruben and Kamen in 1940 were able to demonstrate the fixation of carbon dioxide by a non-green tissue, that of the barley root.

The reactor has completely altered the position and the biologist has now available, and in quantity, such isotopes as carbon-14, nitrogen-15, phosphorus-32, sulphur-35, cobalt-60, iodine-131, and even hydrogen-3. The use of tracer elements is now so widespread that even some time ago private firms in U.S.A. had made available over 300 labelled components. From these, of course, other labelled substances required for experimental work can be built up, either by purely chemical means or even by biosynthesis, as, for example, by feeding a plant with $^{14}\text{CO}_2$ and extracting from the leaves labelled carbohydrates. In a similar fashion, labelled alkaloids can be obtained, and, by fermentation, labelled fatty acids. The long half-life of carbon-14, namely 5,720 years, makes it of great value not only to biologists but also to archaeologists in dating organic remains. In contrast, cobalt-60 has a half-life of only about 5 years, sulphur-35 of 87 days, phosphorus-32 of 14 days, and iodine-131 of 8 days.

Filter-paper partition chromatography. This is a special type of liquid-liquid partition chromatography which was developed by Martin and Synge from their work on the separation of amino-acids. They had used water-saturated silica gel as the stationary phase and an organic solvent as the mobile one, but they ran into certain difficulties; later, in 1943, they found cellulose suitable as a supporting medium. From this came, in 1944, a full description of filter-paper partition chromatography, a technique now so widely used, not only in organic and inorganic chemistry, but also in physiological studies generally. The methods evolved by these two workers have been described as probably unique, not only by reason of their simplicity and of their elegance of conception, but also by reason of the wide scope of their application.

I may say at this point—though the protest almost certainly comes too late—that the abbreviated terms in which this technique is often described, such as 'paper chromatography' or just 'chromatography', seem most unfortunate. For such terms refer only to the colour aspect of the method, whereas what the liquid-liquid paper system brings about—and in this lies its great value—

is a *partition*, a separation of substances; the colour effects obtained by the use of various reagents only demonstrate the separation and are secondary. One now even finds authors of scientific papers referring to *chromatographic separation*, whereas the production of colour (chromatography) has nothing to do with the separation and comes after it.¹ Could not the word *partition* always be included in the title given to this particular technique? It is noteworthy that in the preliminary description of the method given by Gordon, Martin, and Synge in 1943, and in the full descriptive paper by Consden, Gordon, and Martin in 1944, the technique is described as 'Partition Chromatography' or as a 'Partition Chromatographic Method'.

Combination of techniques. One of the great advantages of the filter-paper partition technique is the ease with which it can be combined with other methods. Thus in this year's volume of the Society's *Journal of Experimental Botany* there is an example of the association of this technique with bioassay in the study of plant-growth substances. After the separation of the auxins in different zones of the paper had been demonstrated by spraying with a suitable reagent, the growth substances were extracted from appropriate areas of similar, but unsprayed, paper and their physiological activity tested by the coleoptile-extension techniques.

Isotope-tracer methods and paper-partition chromatography. It is doubtful which of these techniques can be claimed as giving the greater experimental power to the biologist, but when they are combined, in what has been termed, and so inadequately, radiochromatography, they provide what to an older biologist like myself seems a tool of extraordinary precision.

The first employment of these two techniques in combination was in animal physiology, when, in 1947, Fink, Dent, and Fink used iodine-131 and the paper-partition method in the study of thyroid extracts; since then the use of the combined techniques has been wide.

The remarkable physiological results to be obtained by the combination of isotope labelling and paper partition is shown by a series of papers from the Radiation Laboratory of the University of California which have appeared in the Society's botanical journal from 1950 onwards. Using carbon-14, the products of photosynthesis can be studied in algae after an exposure to light of only 2 minutes. The wide range of results that can be obtained by this association of techniques is shown in the latest paper of the series in which 27 different plants were studied, including many algae, a liverwort, 2 mosses, 2 ferns, and 9 flowering plants. It was found that after an exposure to light of only 5 to 10 minutes the carbon-14 of the assimilated carbon dioxide had been transferred to over 20 organic substances; these included 7 sugars, 6 amino acids, 7 organic phosphates, and also some organic acids and some unknown substances.

Again, an illustration of the variety of plant-physiological studies to which these techniques can be applied is to be found (in the same number of the

¹ Surely, also, the proper description of a later development of this method is 'two *directional*' not 'two *dimensional*'.

Journal of Experimental Botany) in an account of a much narrower investigation, but one giving results, of their kind, equally illuminating. By presenting to destarched leaf-disks, floating on a nutritive solution, various sugars labelled with carbon-14 (including sucrose with either the glucose or the fructose so differentiated) much light was thrown on a problem which was an active one when I was a student, namely that of the precursor of starch in the green leaf.

Paper electrophoresis. Another comparatively new method of separation of related substances, such as proteins, in which paper is employed is paper electrophoresis, where the substances move in the paper under the influence of a potential gradient in the electrolyte saturating the paper. I may say, in passing, that it has been claimed recently that from the degradation products of tobacco leaf-mosaic in alkaline solution there can be separated, by this type of electrophoresis and by centrifugation, a material which, though a degradation product, is nearly as biologically active (i.e. as infective) as the original virus.

Polarography. In connexion with electrophoresis, the polarographic method of analysis may be referred to, since it dates back to 1922, a little before the foundation of the Society. The method has the advantage of being quick and easy and requiring only small amounts of material. Oxygen is readily reduced at the dropping mercury electrode, and the method has been employed for the determination of oxygen in solution in studies of photosynthesis and of respiration.

The microscope. The microscope can hardly be called one of the biologist's experimental tools, but it is certainly an analytical tool of the greatest value. Of recent years there have been such developments as the phase-contrast microscope and the interference microscopy. Of the *electron microscope*, and of its power of revealing material particles far smaller than can be observed by means of light rays, little need be said. In the Society's botanical journal a number of papers have appeared which prove how much this instrument can display of the minute structure of the cilia of motile cells of plants. Also the contributions to our knowledge of viruses resulting from the use of this type of microscope are well known. I may mention that recently (*Nature*, 26 March 1955) some very striking electron micrographs of fractions of tobacco-mosaic virus (at a magnification of 150,000) have appeared from the Max Planck Institute in Tübingen.

X-ray diffraction analysis. This method of analysis is the working tool of the physicist and chemist rather than of the biologist. Perhaps the investigation by this method of the molecular structure of deoxyribonucleic acid, which is being carried on so actively at the present time, is of the most interest to the biologist. The helical nature of the molecule seems to have been established. As a further development one may notice that the scattering of neutrons is now being used in the location of atoms in single crystals, and that by this means the position of the hydrogen atoms in α -resorcinol has been determined.

General laboratory conditions. Younger biologists must find it difficult to imagine conditions of work in laboratories where refrigerators and centrifuges were little used and thermocouples rarely employed. Laboratories, also, in which the concept of hydrogen-ion concentration, and the concomitant use of buffers, was not yet available, and in which the employment of photo-electric cells and electronic devices generally was yet far off. The Warburg respiration apparatus is also a development of the last three decades, coming in, I believe, about 1926.

Standard apparatus. Of instruments to measure growth, the humidity of the air and, what is now called, the porosity of the leaf there have been great developments. In a recent number of the *Journal of Experimental Botany* there is a description of an auxanometer which will record increments as small as 0.03 mm.; the growing point of the plant carries a platinum strip which exerts a pressure of only 2 mg., while sparking is prevented by the use of a valve-operated relay.

Hygrometers. In 1938 (vol. 15, *Journal Exp. Biology*) there is a description of a hygrometer, employing a single human hair, which is delicate enough to demonstrate the existence of a humidity gradient up to 1.2 cm. above a rapidly transpiring leaf in slowly moving air. In 1951 (*J. Exp. Bot.* 2) it is shown that by taking advantage of the Peltier effect a thermocouple can be used as a delicate dewpoint hygrometer.

Porometer. Since its inception in 1911 by Darwin and Pertz the porometer (which measures the viscous flow of air through the leaf) has been steadily improved in both rapidity and ease of use. The original one was followed in 1915 by that of Knight; and this again was succeeded in 1934 by the Resistance Porometer of Gregory and Pearse. To the last-named type modifications have been introduced in 1941 by Heath, and in 1951 by Spanner and Heath and by Heath and Russell.

Communication between scientific workers by the printed word. As an editor of a scientific journal for twenty-five years, one method, though not a laboratory one, is to me of especial interest, namely the method of communication between one scientific worker and another. In commenting on the paper-partition technique I have referred to the unsuitability of the terms often used in referring to the method: terms which not only show an insensitive use of language, but which, in effect, conceal the nature of the technique from anyone unfamiliar with current scientific jargon. In these modern days when fields of scientific inquiry so markedly overlap it would seem most unfortunate if co-operation between, say, the biologist, the chemist, and the physicist is to be hampered by a terminology which is not as easily understandable as it could be made to be.

One slight cause of confusion in science is the use of the same word in entirely different meanings. The *nucleus*, for example, has to biologists meant the *cell* nucleus since the work of Robert Brown in 1831, but to the physicist it now means something entirely different. Thus when the word appears without a prefix in the title of a scientific communication one is often left in

doubt as to whether the subject-matter is biological or physical. Again, *plasma* has a well-defined meaning in animal physiology, especially in relation to the blood. Last year, however, I came across a communication relating to physics which bore the title—surprising to a biologist—‘Non-isothermal plasmas in the rare gases’.

Leaving on one side these minor confusions, I would assert that during the last few decades there has been a definite deterioration in the use of the English language in scientific journals. Some of it is due, no doubt, to the increased tempo of modern life and the desire for rapid publication, and some also to the malign influence of newspaper headlines, where the word which fits the space is more important than the word which is most apt to the meaning. Much of it, I am sure, is a refusal to take the trouble to find the words which express as precisely as the author can achieve what he is attempting to communicate. What results is very often not even the second best but something much below it. In a recent number of *Nature* there was to be found the expression ‘un-outgassed electrodes’, and also the following six words strung together without a comma or a hyphen: ‘excellent square wave modulated returned signals’. I would add two other titles which I have noticed in recent journals. The first, an example of the omission of a word, is ‘Coagulation Mechanism in the Horse’, which calls up a mental picture of the animal coagulating as a whole. The second (also from *Nature*) is a communication with the title—‘A Simple Automatic Fraction Cutter’. This leaves one wondering whether the instrument cuts up something (so curiously termed a ‘fraction’) in the way a bacon-cutter cuts up bacon; or whether the instrument cuts up something *into* ‘fractions’. The text, however, shows that the reader has been entirely misled; the apparatus is in no way designed for *cutting* in any ordinary sense, but is one for the distribution of a liquid in equal portions into separate test-tubes.

I have time to record an objection to only one other literary usage or misusage: that which describes something as ‘so many times greater than’ another. This expression is common in the lay press and is sometimes—reprehensively in my view—to be found in scientific journals and in scientific broadcasts. It would seem evident that if something is x times *greater* than another it is $x+1$ times *as great*. It is sometimes apparent, however, from evidence in the text that a scientific author is using ‘greater’ and ‘as great’ as interchangeable terms; without such evidence an ambiguity remains wherever ‘times greater than’ is to be found.

I was once examining a Ph.D. candidate who had stated in a thesis—the candidate was a woman—that some quantity was ‘three times greater than another’ when what was meant was three times as great. I tried to convince her of the error of this usage by pointing out that if ‘three times greater’ meant ‘three times as great’, then ‘two times greater’ must mean ‘twice as great’, and *once greater* must be equality, that is, *not greater* at all. The logic of this, however, passed completely over her head, for the only response I obtained was: ‘I don’t say two times greater.’

I realize that this brief survey of some changes during the past fifty or sixty years in biological outlook and in experimental-botanical techniques—in which I have rashly included some reference to the scientific man's method of communication with his fellows—is a very inadequate and a very uneven one. Another biologist would have stressed other aspects and other technical changes. I hope, however, that what I have said may remind members of the great alterations in plant biology that have taken place during the past half-century, and of the great variety of experimental tools that the investigator of today has, so enviably, at his command.



Localization of Nucleic Acid Synthesis in Root Meristems

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Received 11 October 1955

SUMMARY

Adenine-8- C^{14} was supplied to roots of *Vicia faba* and *Allium ascalonicum* and its incorporation into DNA was studied from autoradiographs of hydrolysed sections. These roots have a quiescent centre to the meristem where the cells do not synthesize DNA and probably, therefore, play no part in the construction of the root. The boundary between the quiescent centre and the central cap initials is clearly defined and this suggests that there is as little cell interchange between the histogens as there is in roots with visibly discrete histogens.

INTRODUCTION

It was shown for *Zea* that there is a quiescent centre to the root meristem (Clowes, 1956). It lies at the pole of the stele and epidermis-cortex complex and extends to about one-third of the width of the root. Its cells have a lower RNA content and a much lower rate of DNA synthesis than are found in the surrounding cells, and it was proposed on this evidence and on structural grounds that the cells divide rarely or never. They are surrounded by cells which do divide. The boundary of the quiescent centre is sharply defined on the distal side where it touches the root cap initials, but ill defined on the proximal side.

Roots of grasses like *Zea* are peculiar in that their histogens appear discrete. The cap is especially well demarcated from the rest of the root by the thickness of the dividing walls which is ascribed to early pectinization in the embryo by Guttenberg, Heydel and Pankow (1954a). A quiescent centre might be related to the discreteness of the histogens, for its shape as revealed by phosphate and adenine incorporation in DNA fits exactly with a quiescent centre postulated on mechanical grounds. In other roots where the histogens do not appear discrete there might be interchange of cells between tissues, and that would involve cell division and DNA synthesis in the part of the apex corresponding to that which is quiescent in *Zea*.

On the other hand, there is some evidence from surgical experiments that this part of the apex is not important in tissue formation (Clowes, 1953). This view is not supported by other workers. It is not possible to investigate other kinds of root anatomically as completely as can be done for grass roots (Clowes, 1954) because one cannot be sure that estimates of the number of cells interchanged between tissues are good. But now the techniques used to

measure DNA synthesis in *Zea* roots can be applied to more difficult meristems and may help to settle the crucial point of cell interchange.

METHODS

The plants are grown with their roots in a solution of adenine labelled with C^{14} at position 8. After one, two, or three days the roots are fixed, sectioned at 6μ , hydrolysed in N HCl at 60°C . for 10 minutes to remove the RNA, and covered by a photographic emulsion. After being exposed to the β particles for 28 days the autoradiograph is developed, stained, and mounted in balsam.

The nutrient solution is made up to an activity of 0.2 mC./l. with adenine which has a specific activity of 1 mC./mM. The solution is filtered through fine sintered glass into sterile bottles when not used immediately. Film stripped from Kodak autoradiographic plates was used as described by Doniach and Pelc (1950). Many fixatives and stains are unsuitable because they reduce the sensitivity of the emulsion and spoil its transparency. The best fixative tried was acetic alcohol, though this is not ideal for sectioning, and the best stain was the mixture of Leishman and Giemsa used by Lajtha (1954a) for blood.

The usefulness of the DNA synthesis measurement here depends upon the fact that the amount of DNA in a cell remains fairly constant except when it is doubled before mitosis. The original work of Howard and Pelc (1951, 1953), using P^{32} to label the DNA, determined the time of DNA synthesis in relation to the onset of mitosis, and their results have been confirmed by independent methods (Thoday, 1954). Lajtha (1954b) used adenine labelled with C^{14} and showed that the labelling of human bone marrow cells grown *in vitro* was confined to the DNA after acid hydrolysis and washing. The rate of RNA turnover in the nucleus is higher than in the cytoplasm and so the autoradiograph over the nuclei can be a good estimate of DNA synthesis only if RNA is removed. Lajtha has shown that acid hydrolysis is as good as ribonuclease hydrolysis, and it is much more convenient.

Adenine is readily taken up by roots from aerated solutions. The period of immersion allows the cells time to synthesize DNA after their last mitosis and it is found that meristematic cells produce strong nuclear autoradiographs, whereas other cells do not. There are normally some silver grains over nuclei of non-meristematic cells even in hydrolysed roots, but their number is one-third or less of the number over meristematic interphase nuclei. Their presence is due to the unavoidable background and probably also to interchange unaccompanied by increase of DNA. However, by comparing the nuclear autoradiograph over all parts of the section, it is possible to obtain a satisfactory measure of rates of DNA synthesis.

RESULTS AND DISCUSSION

A Dicotyledon and a Monocotyledon were chosen to provide the most instructive examples of meristems without visibly discrete histogens. The

first, *Vicia faba*, has a meristem in which none of the histogens is readily distinguished (Fig. 1) and there is no adequate account of how its pattern of cells is produced because it is impossible to work out how the cells divide and grow on anatomical data. An account of the controversy over this type of meristem is given by Popham (1955).

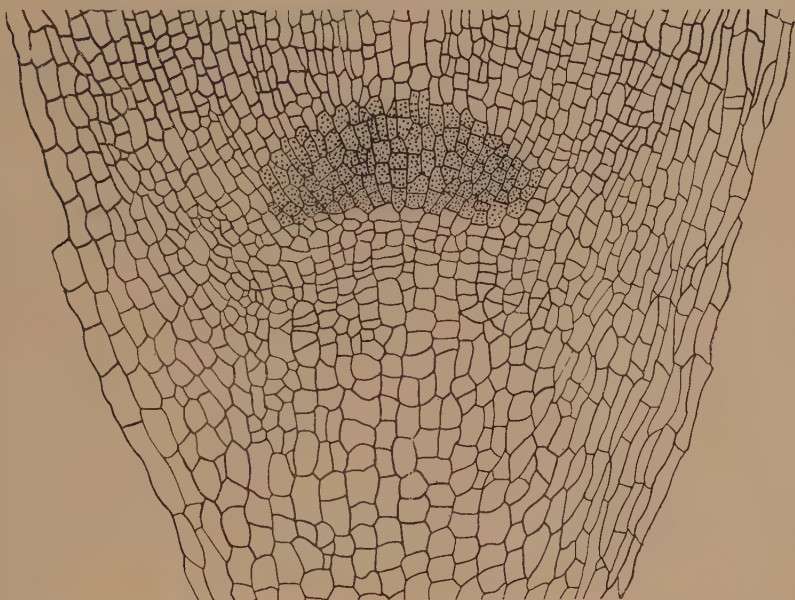


FIG. 1. Median section of seedling tap root apex of *Vicia faba*. The position of the quiescent centre is indicated by dots. $\times 120$.

The autoradiographs of roots supplied with adenine-8- C^{14} show a quiescent centre as in *Zea* extending for a third or a half of the width of the root (Plate 1a and Fig. 1). The quiescent centre is marked by the absence of any strong nuclear autoradiograph and, as in *Zea*, its boundary is sharp on the distal side and indefinite on the proximal side. There is a band of cells across the root with strong nuclear autoradiograph just below the quiescent centre. This band obviously corresponds to the line of cap initials and their derivatives on the distal side in *Zea*. The longitudinal files of cells in the cap of *Vicia* with its broad columella run up into the stele and cortex and there is no visible demarcation between the tissues as there is in *Zea*. This has led some anatomists to hold that the promeristem could be reduced to a single cell functioning as a totipotent apical cell (Guttenberg, 1947). Others think that a transverse meristem at the level of the band of meristematic cells below the quiescent centre gives rise to both cap cells on the distal side and stele and cortex cells on the proximal side (Popham, 1955). But the present work demonstrates that both these views are unlikely to be true. For in the first place the promeristem must consist of all the cells on the surface of the quiescent centre. Secondly,

there would not be a quiescent centre at all if the transverse meristem added to the cells of the stele and cortex unless the proximal daughters of the transverse meristem stopped dividing and then started dividing again after they had been displaced to a position several cells away from the transverse meristem: and if this occurred it would not be so easy to account for the continued synthesis and division on the flank of the quiescent centre, and also for the sharpness of the boundary of the distal side of the quiescent centre.

In describing the structure of *Fagus* roots (Clowes, 1950) it was suggested that the cytogenenerative centre was a 'cup-shaped' promeristem and this shape has been accepted by Guttenberg, Heydel and Pankow (1954b) for other roots. At that time it was assumed that the cells at the pole of the stele were meristematic, and that the base of the inverted cup, therefore, consisted of the columella initials and a contiguous line of stele and cortex initials. It was shown in a series of surgical experiments on both *Fagus* and *Vicia* (Clowes, 1953) that the cytogenenerative centre must be broad and not confined to one or a few axial cells as proposed by Brumfield (1943) and Guttenberg (1947). Its width was consistent with the cup-shaped promeristem hypothesis and there was no reason then to suspect that some of the cells were quiescent. The present work suggests that the promeristem is shaped like a hollow lens rather than a cup, but it gives additional evidence for the broadness of the promeristem.

The root of *Allium ascalonicum* differs from that of *Vicia* in that the epidermis-cortex complex is more distinct from the peripheral part of the cap. This root is also narrower and its columella is much narrower, being three or four cells across instead of about ten. However, the files of columella cells sometimes appear continuous with those of the stele, and Guttenberg, Heydel, and Pankow (1954b) believe that there is a cell which is genetically common to both tissues in a similar species of *Allium*. It is true that their view is not inconsistent with the arrangement of cells at the apex, but there are difficulties in interpreting the apex in this way. It is unlikely that *Allium* differs so greatly from *Zea* or *Vicia*, and secondly Steffen (1952), working on *Impatiens*, was able to criticize Guttenberg's central cell theory because at no stage in the embryo root is there a cell common to all tissues.

Autoradiographs of roots supplied with adenine show a quiescent centre within the meristem just as clearly as in *Vicia* or *Zea* and there is a line of columella initials demonstrated by the prominent nuclear autoradiographs (Plate 1b). The quiescent centre occupies a smaller proportion of the volume of the apex than in the larger roots. This is to be expected because the width of the quiescent centre depends mechanically upon the width of the columella rather than upon the width of the root in this kind of meristem (Clowes, 1954). The existence of a well-demarcated line of cap initials is good evidence against the common origin of cap and stele cells in normal root growth.

In those Pteridophyte roots with apical cells it has long been suspected that the apical cell divides less frequently than its daughters. It might be thought that the quiescence of the meristem pole proposed for Angiosperm roots was similar to the low rate of division in apical cells. If this were true there should

be little or no nuclear autoradiograph over apical cells in roots treated like those of *Allium* and *Vicia*. But in the adventitious roots of *Azolla* this is found not to be so, for the nuclear autoradiograph is as prominent over the apical cell as over the rest of the meristem. Moreover the cytoplasm is as basophilic as in the other dividing cells although vacuoles are more conspicuous.

There may be some physiological cause making the region at the pole of the histogens less active in producing new cells than the rest of the meristem, and this cause may account for both the quiescent centre of Angiosperm roots and the slower growth of apical cells in the smaller Pteridophyte roots. But there also appears to be a quantitative difference between the two in that, under similar conditions, an apical cell does synthesize DNA and a quiescent centre does not. The difference may be related to the difference in width of the roots.

In *Vicia* and *Allium* as in *Zea* it cannot be said that no divisions occur in the quiescent centre, for its shape, size, and position in the meristem probably fluctuate. All that can be said is that in normal growth the quiescent centre is not important in producing new cells for the histogens, and this means that there is little or no cell interchange between the histogens. The model which fits the observed behaviour in the apex most easily is one in which initial cells over the whole surface of the quiescent centre divide so that the inner daughter cells remain meristematic and the outer daughter cells differentiate after further divisions. In this model the shape of the cytogenenerative centre is essentially similar to the outline of an apical cell in Pteridophyte roots.

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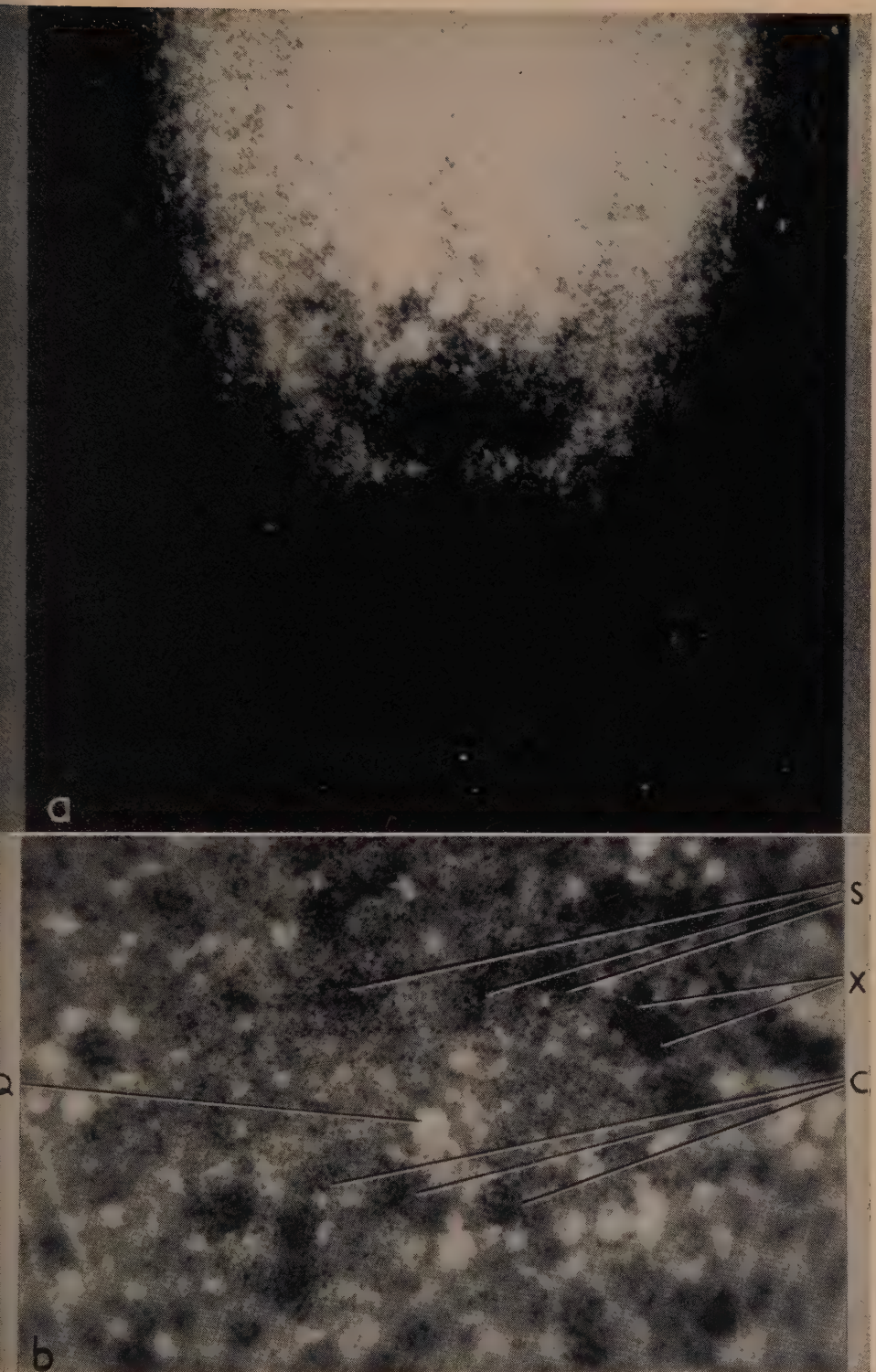
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EXPLANATION OF PLATE

Plate 1a. Autoradiograph of a hydrolysed median section of a tap root apex of *Vicia faba* supplied with adenine-8- C^{14} for one day. Photographed by dark ground illumination to resolve the silver grains over the large field. The apparent size of the silver grains (white) is greatly exaggerated by the diffraction halos. The cap initials and their daughters show as a white line below the black quiescent centre. The tip of the cap extends to the bottom of the photograph. $\times 150$.

Plate 1b. Autoradiograph of medium section of adventitious root apex of *Allium ascalonicum* supplied with adenine-8- C^{14} for one day. The silver grains (black) are mainly grouped over the interphase nuclei of meristematic cells. Q, quiescent centre; c, cap initials; s, stele initials; x, cortex initials. $\times 400$





Studies in Stomatal Behaviour

VII. EFFECTS OF ANAEROBIC CONDITIONS UPON STOMATAL MOVEMENT—A TEST OF WILLIAMS'S HYPOTHESIS OF STOMATAL MECHANISM

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SUMMARY

An experiment was carried out to investigate stomatal responses in wheat to four 'closing treatments', viz. high carbon dioxide concentration, darkness, dry air and nil, each given under both aerobic and anaerobic conditions. Thus the effect of lack of oxygen on the closing (or opening) tendency was estimated. Changes in $\frac{3}{\lambda}$ (conductance) calculated from resistance porometer readings were used as data and reasons are given for thinking this is the best available measure for investigating stomatal dynamics in wheat. Williams's hypothesis demands that lack of oxygen should cause stomatal opening or prevent closure; the present experiment shows that anaerobic conditions significantly increase the closing tendency when 'closing treatments' are first applied. There is also some suggestion that oxygen-lack itself tends to cause closure in the absence of any other 'closing treatment'. Williams's hypothesis in its original form is thus disproved (for wheat) but the present results would be consistent with an 'active' uptake of water by the guard cells contributing to stomatal opening. A nearly significant interaction between carbon dioxide and oxygen suggests that under anaerobic conditions a 'closing substance' may perhaps be formed, for example, by the union of some intermediate in glycolysis with carbon dioxide.

INTRODUCTION

THE new hypothesis for the mechanism of stomatal movement put forward in a recent lively and stimulating paper by Williams (1954) deserves serious attention even though, as in the case of the equally ingenious hypothesis produced by Scarth (1929), there is no direct evidence to support it. Contractile vacuoles have not yet been recorded in stomatal guard cells, but recent work on 'active transport of water by mitochondria' gives rise to intriguing speculations as to whether these may be the seat of the necessary 'pumps', as they are apparently of so many important activities in the cell. Price and Davies (1954) found that *in vitro*, rat liver mitochondria would, under conditions favourable to oxidative phosphorylation, extrude water which had been taken up when ATP and AMP were absent, oxygen uptake being high in both cases. A further elaboration of the hypothesis would be needed to make the 'pumps' directional and this might perhaps be provided by their movement bodily from one part of the cell to another. Rapid movement of mitochondria occurs with protoplasmic streaming such as may be

seen in onion stomata (Heath—unpublished). Obvious objections to the suggestion that stomatal closure is caused by mitochondria picking up water near the tonoplast, transporting it to the dorsal wall of the guard cell and there discharging it are: (a) the small volume of mitochondria relative to the change of cell volume probably needed and (b) the resistance of the plasma membranes to water movement which would tend to make the 'pumps' inefficient—the local increase of solute concentration near the vacuole and decrease near the cell wall tending to even out by diffusion within the cytoplasm instead of by osmotic transfer of water through the membranes.

A simpler hypothesis would be merely to suppose that the mitochondria, by extruding water, lowered the suction pressure of the guard cell as a whole and so caused closure. They would then function much like Scarth's postulated amphoteric colloids, which would of course have been equally effective in the cytoplasm instead of in the vacuole where he supposed them to be. Here, however, the objection on the grounds of the small volume of mitochondria would appear even more cogent.

Not only the above but any 'active'¹ excretion of water out of the guard cells to cause closure would presumably need oxygen, as mentioned by Williams. He cites the evidence of Freudenberger (1940) in favour of his hypothesis, but this does not appear admissible. Her experiments, with leaves in continuous darkness, showed in most cases a closing response to O₂-free nitrogen. Closure continued for from 1½ to 4 hours and was then succeeded by opening. Williams quotes with apparent approval her opinion that the closure was doubtless a normal reaction, carried out by virtue of the oxygen still remaining in the intercellular spaces. Since, however, she had no control leaf area given oxygen throughout it is impossible to say with any confidence what would have been the 'normal' reaction. In some cases, the stomata were *opening* in carbon dioxide-free air (in darkness) when the oxygen-free nitrogen was applied. The latter treatment must immediately have *reduced* the oxygen supply but why this should reverse the opening trend and cause a closure is far from clear on Williams's hypothesis; furthermore it seems inconceivable that with the stomata not fully shut any appreciable concentration of oxygen could remain in the leaf for a matter of hours. The very much delayed reopening in nitrogen found by Freudenberger can scarcely be considered relevant in view of the absence of a control.

The hope expressed by Williams that his hypothesis would stimulate new experimentation, coupled with the unsatisfactory nature of Freudenberger's evidence, led us to design an experiment to test the effect of deprivation of oxygen upon the closing responses of stomata. This appears to be the most crucial point at which the new hypothesis can be tested.

¹ The term 'active' is here used in the same sense as by Williams who postulates: '... an "active", non-osmotic, energy-requiring transfer of water from guard-cell to neighbouring cells, possibly mediated by contractile structures of some type'. According to Spanner's (1954) definition contractile vacuoles would result in passive movement of water under pressure gradients and active water movement (e.g. by electro-osmosis) would not necessarily require oxygen.

EXPERIMENTAL

Williams appears to postulate that all stomatal closure is due to 'active' excretion of water from the guard cells, stimulated (at least in the dark response) by high carbon dioxide; in the absence of such 'active' excretion (low carbon dioxide conditions) opening occurs by osmotic intake of water. It was therefore decided to apply various 'closing treatments', and as the most important comparison was the difference in response to these in the presence and absence of oxygen, each closing treatment was to be given under aerobic and relatively anaerobic conditions simultaneously on two different parts of the same leaf; thus a comparison of the slopes of the two time curves would indicate the effect of oxygen on the closing (or opening) tendency, unaffected by leaf to leaf differences or any diurnal rhythm. The treatments are summarized in Table I.

TABLE I
Summary of treatments

Sub-treatment: 'oxygen supply' (within leaves)	Main treatment (between leaves)			
	R.H. at 25° C.	CO ₂ conc.	Light intensity	'Closing treatment'
Air (20% O ₂) } N ₂ (0.0% O ₂) }	75%	0.00%	600 f.c.	Nil
Air (20% O ₂) } N ₂ (0.0% O ₂) }	75%	0.10%	600 „	CO ₂
Air (20% O ₂) } N ₂ (0.0% O ₂) }	75%	0.00%	0 „	Dark
Air (20% O ₂) } N ₂ (0.0% O ₂) }	0% (approx.)	0.00%	600 „	Dry

Replicated on Days I, II, and III.

Potted plants of 'Charter' wheat were used and the two pairs of leaf chambers used by Heath and Russell (1954, *a, b*) were attached to the 'flag' leaf or the previous leaf; they were illuminated from above with a constant light intensity of 600 f.c. in the constant temperature chamber used by those authors, which was maintained at 25° C. The leaf chambers of each pair (lower and upper) were swept in series, between readings, with a constant flow of 4 l./hour of gas, at a pressure of 20 cm. of water above atmospheric. Thus a small flow of gas must have passed into the leaf through the stomata within each of the chambers and escaped laterally. This sweeping of the intercellular space system was intended not only to condition the stomata but also, coupled with the fact that the two pairs of chambers were 2 cm. apart, to prevent the gas used for one pair from affecting the leaf area in the other by diffusion within the leaf. Four gas supplies were used, all from cylinders and controlled by pressure regulators, needle valves, and flowmeters, viz. (1) carbon dioxide-free air, which was obtained by scrubbing ordinary cylinder air with

'Sofnolite'; (2) Carbon dioxide-free and oxygen-free nitrogen,¹ similarly scrubbed; (3) air containing 0.1 per cent. carbon dioxide; (4) oxygen-free nitrogen containing 0.1 per cent. carbon dioxide. After being humidified the gas streams entered the constant-temperature plant chamber and there passed through saturated sodium chloride solution to bring the relative humidity to 75 per cent.; they were then passed to the appropriate leaf chambers by a suitable arrangement of taps. They finally escaped into the plant chamber by bubbling through 20 cm. height of water, thus maintaining its humidity somewhere near saturation.

For porometer readings the connexion between the lower and the upper chamber was closed, the former being swept with gas at a pressure of +20 cm. of water as before and the latter connected to atmosphere via a capillary resistance of about one Gregory and Pearse unit (Heath, 1939). Two manometers showed the pressure drop across the leaf from lower to upper chamber (P_l) and that across the capillary (P_c) respectively. The leaf resistance was calculated as P_l/P_c , the value of the capillary resistance being taken as unity. No correction was made for the small difference (11 per cent.) between the two capillaries used for the two pairs of leaf chambers, nor for any differences in leaf area or stomatal numbers. Such corrections are not needed if attention is confined to the slopes of the curves for log (leaf resistance) as they would only affect the levels and not the slopes of such curves; for curves of $\frac{3}{2}$ /(conductance) (see Discussion) they would have a small effect on the slopes, but the errors due to their omission should be random. The necessary taps were placed within the constant temperature chamber to avoid condensation, and were operated without opening the chamber by a system of threads attached to their handles. Porometer readings occupied about 2 minutes and were taken at 15-minute intervals.

The experimental procedure was as follows: after attaching the leaf chambers, carbon dioxide-free air at 75 per cent. R.H. was passed through both pairs for a preliminary period. When both leaf areas showed a somewhat similar porometer reading, carbon dioxide-free and oxygen-free nitrogen was substituted for the air passing through one pair of chambers. Fifteen or 30 minutes later one of the four 'closing treatments' (Table I) was applied to both areas simultaneously and porometer readings were continued at 15-minute intervals as before. The 'closing treatments' were obtained as follows:

1. *Nil*. The carbon dioxide-free air and nitrogen were continued as before (in light and at 75% R.H.)
2. CO_2 . Air or nitrogen containing 0.1 per cent. carbon dioxide were substituted for the carbon dioxide-free gases.
3. *Dark*. The whole plant chamber was darkened.
4. *Dry*. The humidifiers were disconnected and the carbon dioxide-free gases passed through pure glycerine instead of saturated sodium chloride solution.

¹ B.O.C. Less than 10 p.p.m. oxygen.

The four closing treatments were carried out in random order during a day, a new leaf being used for each to avoid possible after-effects. They were replicated on three days. The design thus resembled a randomized block layout with split plots; 'Days' corresponding to blocks, leaves to main plots with 'closing treatments' as main treatments and the two pairs of leaf chambers to sub-plots with air or nitrogen as sub-treatments. The changes of $\sqrt[3]{\text{conductance}}$ and also of $\log(\text{resistance})$ over the first, third, and fifth 15-minute periods following application of the closing treatment were used as data, thus measuring the closing or opening tendency during those periods. The second and fourth 15-minute periods were omitted from the analysis to avoid the lack of independence which would otherwise result from using single values twice over in calculating changes for successive periods. In the case of the 'Nil' treatment the first 15-minute period was taken as beginning 15 minutes after the application of nitrogen to one pair of leaf chambers, this being the time when the other treatments were applied in most cases.

RESULTS

Three mishaps occurred during the experiment: On the first day in the 'Dry' treatment the taps were inadvertently left in the 'porometer' position throughout the second 15-minute period. Marked stomatal closure occurred in both areas and was followed by opening (again in both areas) during the subsequent 15 minutes (Fig. 1). Here both the 'air' and 'nitrogen' areas were similarly treated and the comparison between the opening or closing tendency in the two should still be valid although the interaction with 'closing treatment' might be affected. Also on the first day in the 'Dark' treatment the humidifier on the nitrogen line became blocked with salt crystals at some time during the first 15 minutes of darkness. The 'nitrogen' area was thus in *still* oxygen-free nitrogen for part of this period, though the residual pressure in the leaf chambers would maintain a flow through the stomata for a time after the blockage occurred. The third irregularity was less explicable and also occurred in the 'Dark' treatment but on the third day (Fig. 2). Following the first application of nitrogen to one pair of chambers the stomata in that area closed so rapidly that after 30 minutes their resistance was about 100 times as great as that in air. It appeared most undesirable to apply the 'Dark' treatment under these conditions and a further half hour in the light was therefore given, with nitrogen fed to the area which had previously received air and vice versa. This caused an abrupt reversal, the stomata opening slightly in the air and closing markedly in the nitrogen to somewhat similar resistances. However, darkening the plant chamber then brought about complete closure in the area now receiving air and wide opening followed by later closure in the nitrogen. The stomata were apparently in a very unstable state and since the alternation of aerobic-anaerobic-aerobic conditions for one area occurred in this replicate only it was thought that these results were not perhaps comparable with the rest of the experiment.

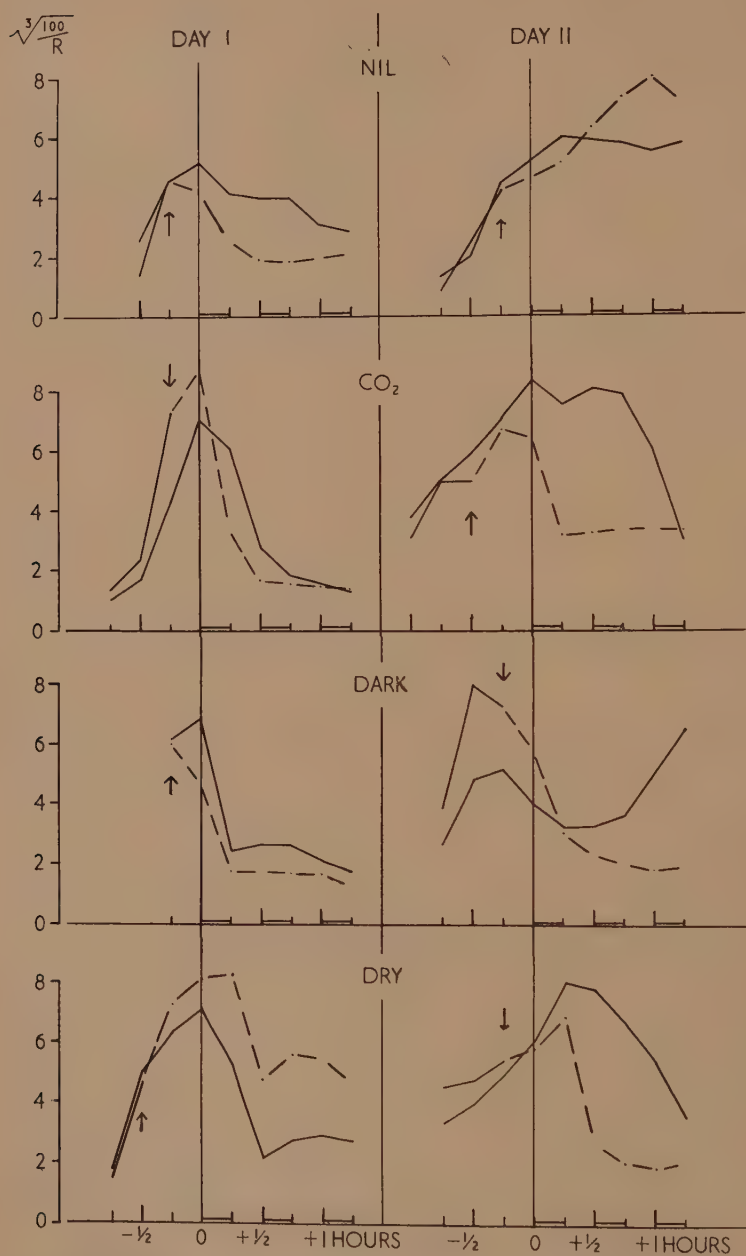


FIG. 1. Time course of $\frac{3}{\text{conductance}}$ in the four 'closing treatments' on the first and second days (15-16. 6. 54). Solid lines denote air and dashed lines nitrogen which was first applied at the times marked by arrows. 'Closing treatments' applied at zero time.

In view of these mishaps in the 'Dark' and 'Dry' treatments the data have been analysed twice: once for the whole experiment and once omitting these treatments throughout, i.e. for the 'CO₂' and 'Nil' treatments only. Only the results of the analyses on $\sqrt[3]{(\text{conductance})}$ will be presented in detail as this is thought to be more representative of stomatal mechanism than log (resistance)—see Discussion.

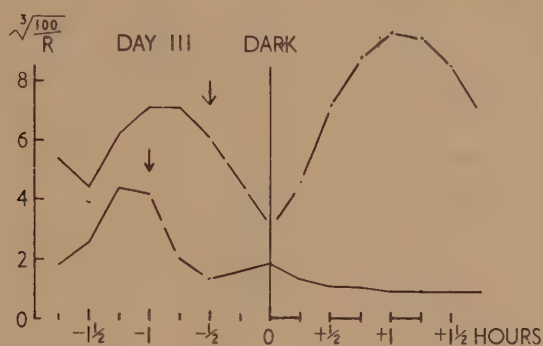


FIG. 2. Time course of $\sqrt[3]{(\text{conductance})}$ in the 'Dark' treatment on the third day (17. 6. 54). Conventions as in Fig. 1.

In both analyses of variance for $\sqrt[3]{(\text{conductance})}$ there were significant average effects of 'Period' ($P < 0.01$), most closure occurring in the first 15 minutes of the closing treatments, of 'Closing Treatment' and of the interaction between these two. These effects are not of particular interest for the present purpose. There was also in both cases a significant two-factor interaction ($P < 0.05$) between 'Oxygen Supply' and 'Period', averaged over all closing treatments. This took the form of a significantly greater closing tendency in nitrogen than air during the first 15 minutes of closing treatment, with a reversal of this state of affairs in the third and fifth 15-minute periods which, however, failed to reach significance by the t test. These results are shown in Table II A (*a* and *b*).

The three-factor interaction between 'Oxygen supply', 'Period', and 'Closing treatment', in the analysis on the 'CO₂' and 'Nil' treatments only, failed to reach the conventional $P 0.05$ level of significance although better than $P 0.10$. Nevertheless the data are presented in Table II B, for if in fact the interaction is a real one it is of considerable interest. The t test cannot legitimately be applied in this case. The apparent interaction consisted of a considerable excess of closure in nitrogen over that in air for the 'CO₂' treatment during the first period and a reversal of this difference in the third and especially in the fifth periods, whereas the 'Nil' treatment showed much smaller differences in the same sense between air and nitrogen. Here there was an appreciable opening in nitrogen during the third period.

It should be noted that the 'Nil', in common with all the closing treatments except 'CO₂', was carbon dioxide-free throughout and thus a large inter-

TABLE II

*Change in $\sqrt[3]{(100/R)}$ per 15 minutes**(+ = stomatal opening; - = closure)**A. Interaction of 'oxygen supply' and 'period'**a. Means over all four 'closing treatments'*

		15-min. periods		
		1st	3rd	5th
N ₂	-1.68	+0.54	-0.10
Air	-0.81	-0.21	-0.39

Significant difference for $P\ 0.05 = 0.84$

b. Means of 'CO₂' and 'Nil' treatments only

		15-min. periods		
		1st	3rd	5th
N ₂	-2.56	+0.48	-0.02
Air	-0.83	-0.12	-0.64

Significant difference for $P\ 0.05 = 1.12$
 „ „ „ $P\ 0.01 = 1.69$

B. Interaction of 'oxygen supply', 'period', and 'closing treatment'

		15-min. periods		
		1st	3rd	5th
CO ₂	{ N ₂	-4.57	0.00	+0.03
	{ Air	-1.77	-0.26	-1.10
Nil	{ N ₂	-0.55	+0.97	-0.07
	{ Air	+0.11	+0.02	-0.18

action between carbon dioxide and presence or absence of oxygen is implied. This is perhaps the most interesting result of the experiment and will be discussed further below. The reversal of the nitrogen-air difference in the fifth period in the 'CO₂' treatment may be attributed to the closure in nitrogen being then more or less completed while the slower response to carbon dioxide in air was still continuing.

Time curves for the four 'closing treatments' on each of the first two days are shown in Fig. 1, while the aberrant result of the 'Dark' treatment on Day III is shown in Fig. 2. In most cases the behaviour in the two areas was very similar until the nitrogen was applied to one. Inspection of all the graphs, including the third day, suggested that in most cases the application of nitrogen then caused either more closure or less opening than in air and the twelve differences N₂-Air for the initial 15 minutes were examined by the t test. Nine of the twelve differences were negative and the mean difference in change of $\sqrt[3]{(\text{conductance})}$ was -0.62 , suggesting a greater closing tendency in nitrogen. The value of t , however, was only 1.74 , corresponding to a probability P slightly above 0.1 . Further, it should be mentioned that in

nine of these twelve cases the end of this initial 15-minute period coincided with the beginning of the first period in the analysis of variance described above. The data in the two tests are not therefore completely independent since some values are being used twice. This lack of independence would, however, tend to cause N_2 -Air differences in opposite senses in the two groups of data, for a chance low value of $\sqrt[3]{\text{(conductance)}}$ at the end of the preliminary 15 minutes would give a tendency towards apparent closure in that period but towards apparent opening in the subsequent one. The fact that in *both* the periods under discussion nitrogen showed a greater closing tendency than air, coupled with the approach to the conventional level of significance in the t test, is suggestive that lack of oxygen not only increases the closing response to 'closing treatments', as shown in the analysis of variance, but also has a closing effect of its own. At least there is no indication that lack of oxygen causes *opening* for the first half hour or so.

The log (resistance) data gave results very similar to those for $\sqrt[3]{\text{(conductance)}}$ but with the three-factor interaction between 'Oxygen supply', 'Period', and 'Closing treatment' significant ($P < 0.05$). This interaction was similar in form to the apparent interaction in terms of $\sqrt[3]{\text{(conductance)}}$.

DISCUSSION

The use of the porometer in dynamic studies to indicate stomatal mechanism, as in the present investigation, raises in its most acute form the question of what function of the readings obtained is the most appropriate. It is salutary to plot the same porometer data both as resistances and as conductances and to note the very different impressions of stomatal dynamics obtained. This particular arbitrary difference does not occur if log (resistance) and log (conductance) are used, as the two curves are then exact mirror images of the same shape and moreover log values are more appropriate for statistical analysis (Heath, 1941, p. 467).

When equilibrium values of, for example, log (resistance) are compared, as in Heath and Russell (1954 *a* and *b*), it is reasonably certain that higher values correspond to more 'closed' states of the stomatal mechanism. When, however, rates of *change* of log (resistance), or any other purely arbitrary measure, are compared the same observed rate of change may well represent very different rates of closing (or opening) in terms of the actual mechanism concerned according to the part of the range in which it occurs. Thus it is quite possible that a change of log (resistance) in one part of the range found to be significantly more rapid than that in another might in fact represent a less rapid change in terms of the fundamental mechanism of the stoma. This might have applied, for instance, to the significant ($P < 0.05$) effect of 'Period' in terms of log (resistance) which indicated more rapid closure in the first 15 minutes, when the stomata were more open, than in the later periods when they were more closed.¹ Such possible errors of interpretation could be

¹ This effect was actually much more significant ($P < 0.01$) in terms of $\sqrt[3]{\text{(conductance)}}$ and therefore was probably real.

largely avoided by confining discussion to differences in rate of change between the N_2 and air areas *in the same period* when the porometer data were usually more or less in the same part of the range, and this course has been adopted even for $\sqrt[3]{\text{(conductance)}}$ which is thought to be a more nearly fundamental measure (see below).

The difficulty of interpreting interactions is, however, the main stumbling-block in the use of arbitrary measures. Thus, for example, absence of significant interaction in terms of \log (resistance), consistent with an hypothesis of additive effects of treatment, is likely to correspond to an interaction of the 'product' type in terms of resistance. It therefore becomes essential to use some measure thought to bear at least an approximately linear relation to the appropriate changes in the stomatal mechanism, so that any interaction or lack of interaction found may represent the effects of treatment on these. The best measure of changes in stomatal mechanism would probably be guard cell turgor; or, especially where humidity effects were concerned, difference in turgor between guard cell and subsidiary cell. It seems likely that the total width across the swollen ends of the two (wheat) guard cells might be a good measure of the latter. The total width in turn might be expected to be at least approximately linearly related to pore width and this has been confirmed for Charter wheat by measurements on 'Stop-action' photomicrographs of stomata on the living leaf (Heath—unpublished). In Heath and Russell (1954, *b*) it was stated: 'There is nothing fundamental about pore width or area . . .' but it seems that this may have been too hasty a judgement as far as wheat stomata are concerned. We have not attempted the calibration of the porometer in terms of pore width for wheat, and in view of the unreliability of Lloyd's method found for *Pelargonium* (Heath, 1950, p. 58) it would seem that this should be done by the difficult method of measurement of stomata on the living leaf. Paetz (1930), however, carried out such a calibration for maize, using a Darwin type porometer and an 'Ultrapak' illuminator, and concluded that 'for not too wide' stomata the porometer rate was proportional to the cube of the stomatal width. Williams (1940) concluded that there were some theoretical grounds for this (see Heath, 1941, p. 457), and the more precise equation given by Heath and Russell (1954, *b*, p. 283) has also been found to confirm the relation to a near approximation, as mentioned below. Plotting, from Paetz's data, $\sqrt[3]{\text{(conductance)}}$ against mean pore width certainly gives a reasonable approximation to a straight line. The maize stomatal pore is similar in shape to that of wheat, and both species have almost equal numbers of stomata on the two leaf surfaces. The relation might therefore be expected to apply for wheat also. Paetz, however, presumably used a permanently attached and unswept porometer cup on one surface only of the leaf and hence differences in stomatal aperture within and above the cup might have been partly responsible for the relation obtained (Heath, 1950). As a check, therefore, the eight values of pore width, given in Table VII, p. 285 of Heath and Russell (1954, *b*) and estimated from the above-mentioned equation, were plotted against cube roots of the reciprocals of the

corresponding resistances. This was found to give a very slightly sigmoid curve which was an excellent approximation to a straight line between 1.6 and 7μ pore width and a reasonable one even from 0.0 to 10.0μ . It was decided, therefore, to use $\frac{1}{2}(\text{conductance})$ in the present investigation as the best measure so far available.

If Williams's hypothesis were correct there should be a very marked opening tendency (or prevention of closure) of the stomata when they were deprived of oxygen, especially in darkness when there could be no production of oxygen by photosynthesis in the guard cells. With the exception of the single aberrant 'Dark' result on the third day the present experiment gives no evidence of such a response, and even in this one case the opening in darkness was preceded by closure in light when nitrogen was applied, which would be difficult to explain on Williams's hypothesis. On the contrary the significant *closing* tendency in response to nitrogen found for the first period as an average over all four 'Closing Treatments' (including the above-mentioned aberrant result) disproves at the $P\ 0.05^1$ level an hypothesis that there is no effect at all; *a fortiori* an opening effect of oxygen-lack would seem to be disproved. This conclusion is to some extent supported by the average closing tendency with nitrogen found in the preliminary period before the application of the 'Closing Treatments', even though this failed to reach statistical significance.

Although Williams's hypothesis in its original form is disproved (for wheat stomata) the results of the present investigation would support the hypothesis of an 'active' oxygen-requiring *uptake* of water by the guard cells, since lack of oxygen tends, at least initially, to accelerate closure or slow down opening. Williams argues that carbon dioxide being very metabolically active may be expected to have a positive effect, but there is no logical reason why it should not operate as an inhibitor and thus low carbon-dioxide concentration might allow an 'active' opening process to proceed. The observation that narcotics cause stomatal closure (Darwin, 1898; Scarth and Shaw, 1951) also lends some support to the view that if an 'active' process is involved it is one of opening rather than closure. The mitochondrial 'pumps' suggested in the Introduction could of course be supposed to work in the reverse direction and cause opening in the presence of oxygen. A relatively high carbon-dioxide supply may be necessary for the formation of a 'closing substance', perhaps organic acid, as suggested below.

The differences between the air and nitrogen areas for the third and fifth periods were perhaps for the most part too irregular to merit discussion, though in the case of the ' CO_2 ' treatment they were suggestive of a greater time lag in air, as mentioned above (p. 320). There was perhaps some tendency to reopen in nitrogen, as in Freudenberg's results but somewhat sooner. Here the possibility of harmful effects of continued anaerobiosis should be borne in mind and these might also account for some of the irregularities observed. Ordinary epidermal cells are apparently in general more easily

¹ $P < 0.01$ if the 'Dark' and 'Dry' treatments are excluded from the analysis (Table II A (b)).

damaged than guard cells and loss of turgor in the former might thus cause stomatal opening after prolonged exposure to lack of oxygen. It seems that the early effects of applying oxygen-free nitrogen are the most likely to supply clear-cut information and the long intervals between readings in Freudenberg's (l.c.) experiments (generally about an hour) add to the difficulty of interpreting her results.

The interaction of oxygen and carbon dioxide, suggested by the three-factor interaction which approached significance in the analysis for the 'CO₂' and 'Nil' treatments, merits some discussion. If real, it indicates that the effects of lack of oxygen and presence of high carbon-dioxide concentration greatly reinforce each other so that their initial combined closing effect is very much greater than the sum of their separate effects (Table II*B*). Until this apparent interaction is better established it would be premature to attach too much importance to a theoretical scheme to account for it, but attempts have been made to formulate such a scheme, as a basis for future experimentation.

In view of the association found by many workers between low pH in the guard cells and stomatal closure, and of the observation of Sayre (1926) that externally applied acetic-acid vapour actually *caused* closure, it seems worth while to consider how carbon dioxide and oxygen might interact to increase acidity in the guard cells. In an earlier paper (Heath, 1950) it was suggested that carbon dioxide could scarcely affect directly the pH in guard cells, which must contain buffer systems, but that the possibility of organic acid formation should not be overlooked. Briefly, then, it may be suggested that some intermediate in glycolysis, perhaps pyruvate, which in the presence of oxygen is rapidly respired in the Krebs cycle to produce carbon dioxide, unites with carbon dioxide in the absence of oxygen to form a 'closing substance'—possibly malate. Accumulation of malate might occur under anaerobic conditions, owing to lack of ATP to allow of its conversion via oxalacetate to phosphoenolpyruvate and so back to pyruvate. Anaerobic conditions, even in the absence of externally supplied carbon dioxide, would then lead to some malate formation owing to the blocking of the Krebs cycle and reaction of the pyruvate with carbon dioxide produced in anaerobic respiration. It is well established for succulent plants that organic acid accumulation is a function of carbon-dioxide concentration, especially at physiological levels (Bonner and Bonner, 1948) and if the guard cells have something resembling crassulacean acid metabolism 0.1 per cent. carbon dioxide might increase malate formation. This effect might be very greatly enhanced by anaerobic conditions with the diversion of pyruvate from the Krebs cycle and its combination with the externally supplied carbon dioxide; thus the interaction would be accounted for. The question of *how* the lowered pH due to malic acid could cause closure would still remain. Indeed, recent work by Mouravieff (1954, *a, b*) indicates that the only effect of low pH on isolated stomata is to cause opening due to imbibition by the cytoplasm, except when the acids are sufficiently concentrated to be toxic. We have obtained no new evidence to justify further discussion of this mechanism at present.

Finally we might consider briefly the possibility that the closing response to oxygen lack is in reality the result of increased carbon dioxide production due to the Pasteur effect. This seems most unlikely to be of appreciable importance in light (Table II A (b) and B) and the apparent interaction between carbon dioxide and oxygen in the first period (Table II B) would take the form that the combined effect was *less* than the sum of the separate effects if in fact both were carbon-dioxide effects.

ACKNOWLEDGEMENT

We are grateful to Dr. J. Edelman of this Institute for advice on the biochemical aspects of the above speculations.

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Studies in the Respiration of Apple at Various Pressures of Oxygen

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Received 24 October 1955

SUMMARY

The results of experiments on the effect of high pressures of oxygen on apples are outlined. It is shown that *partial* pressures of the order of 500 per cent. oxygen are toxic to the tissues and that there is no evidence of an initial increase in CO₂ output over that in 1 atmosphere 100 per cent. oxygen. It is further shown that the *actual* pressure (up to 20 atmospheres) is not responsible for the disorganization of the cells but that there is specific oxygen toxicity.

INTRODUCTION

THE recent appearance of a series of hitherto unpublished papers (Blackman, 1954) and the current interest in 'oxygen poisoning' in tissues (*vide* Stadie, Biggs, and Haugaard, 1944; Dickens, 1946, *a, b*; Barker and Mapson, 1955) have prompted the re-examination of the result of experiments carried out by the author some years ago (1927-9) in the Botany School, Cambridge, upon an aspect of the subject which had not then been studied in any detail.

Over the years a considerable volume of information has been accumulated on the effect of different concentrations of oxygen upon the respiration of various plant tissues (James, 1953). The work here discussed was chiefly concerned with the effect on respiration of oxygen in various concentrations at pressures above atmospheric. The material used was Bramley Seedling apples harvested in October and stored either in a cool apple store or at +3° C. at the Low Temperature Research Station, Cambridge.

It is not proposed to list in detail the literature upon experiments with different pressures of gases, but special reference should be made to the work of Johannsen (1888). He found, *inter alia*, that, with seedlings of *Pisum*, *Helianthus*, and *Hordeum* in air, increasing pressures up to 5 atmospheres caused little or no increase in the rate of CO₂ output compared with that in air at one atmosphere: the rate of CO₂ output was, however, increased by exposure to air at a pressure of 10 atmospheres. These increased rates of CO₂ output, when they occurred, were only temporary. Thus maize seedlings showed an increase in CO₂ output with increasing pressure of air up to about 4 atmospheres, but after some time at high pressures the CO₂ output becomes smaller and smaller until the plant 'approaches death'.

EXPERIMENTAL METHODS

For the experiments here described a phosphor bronze container with a gas-tight top and with a capacity of about 1 litre was designed. A pressure gauge was fitted on to the top of the container. Into the chamber were inserted two small copper pipes, one connected through a series of reducing valves to a pressure cylinder of the gas or gas mixture being used, and the other through a 'gas commutator' to a series of Pettenkofer tubes containing $\text{Ba}(\text{OH})_2$ solution. At the outlet of the valve system on the cylinder was a short tube containing soda lime to ensure that all traces of CO_2 were removed from the gas entering the container. The valve and tap (*T*) (see Fig. 1) made

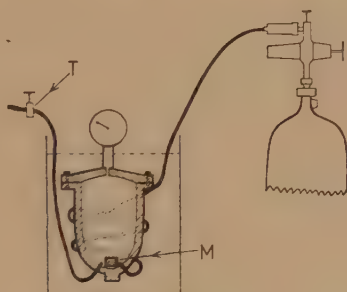


FIG. 1. Diagram of pressure vessel.

possible the control of the gas leaving the container and thus the adjustment of the pressure within the container. The container and the inlet gas pipe were kept submerged in a constant temperature bath at 22.5°C . By the use of batteries of Pettenkofer tubes and a 'Blackman gas commutator' it was possible to continue experiments over long periods so that the tissues could have ample opportunity of attaining the 'adjusted state' of respiratory activity in any concentration or pressure of gas. The apples were put in pairs into the container one above the other on a small stove-enamelled block (*M*) so that as little 'free' space was left as possible. This was important because at 20 atmospheres the amount of gas outside the material would be twenty times the apparent volume. It was found that complete absorption of CO_2 by the $\text{Ba}(\text{OH})_2$ solution occurred with gas flow at 5 l./hour. The normal rate used was, however, 1 l./hour. The gas-stream passed through each of the Pettenkofer tubes for 3 hours before being transferred to the next in the series. Each value given therefore indicates the CO_2 production in mg./100 g. fresh wt./hour for a 3-hour period.

Because of the changes in the physical conditions of the system considerable dilution of the effluent CO_2 was inevitable when the pressure was raised above atmospheric by the temporary increase in the rate of the inflowing gas, and a period of some 24 hours elapsed before the observed rates of CO_2 output represented accurately the rate of production in the tissues.

EXPERIMENTAL RESULTS

A few of the many experiments with apples carried out with the apparatus described above must be given in some detail. Full information on all the experiments is available in the Ph.D. thesis deposited in the Cambridge University Library (Caldwell, 1931).

In Experiment I (see Fig. 2 A) three pressures of 100 per cent. oxygen were used, viz. 1, 6, and 11 atmospheres. The rate of CO_2 output reached a 'steady' level of about 5 mg./hour/100 g. fresh wt. in 1 atmosphere 100 per cent. oxygen while in 6 atmospheres oxygen it rose only to 3.3 mg. and then fell steadily and at 11 atmospheres the CO_2 output fell to zero. It was clear from this last observation and from the fact that the apples were completely brown on removal from the container after a total of 170 hours of experimentation that the CO_2 recorded during the latter part of the experiment was residual CO_2 and not necessarily that produced by the tissues themselves after some hours' exposure to high pressures of pure oxygen. One must not exclude the possibility that the tissues of the apples might retain some substantial amount of CO_2 in solution at high pressures which would be released as the pressure was lowered to atmospheric. In the light of subsequent experiments it became evident that the exposure to 6 atmospheres oxygen was lethal and that no useful information had been obtained by the subsequent increase in pressure.

After it had been realized that high pressures of oxygen were in themselves toxic (e.g. in Experiment XIV, Fig. 2 B) the apples were held for 3 days in oxygen at a pressure of 1 atmosphere (1 atmosphere 100 per cent. oxygen). Increase of the pressure to 15 atmospheres resulted in an immediate decrease in the observed rate of CO_2 output followed by a slight increase for the next 20 hours. A steady fall in the rate of CO_2 output then began and continued to zero despite the return to 1 atmosphere oxygen. Corresponding to the sharp initial decrease in the observed CO_2 output on transition from 1 to 15 atmospheres oxygen, there was a marked increase in the observed CO_2 output for the later transition from 15 to 1 atmosphere. Both these initial changes in the observed rate of CO_2 output following a change of pressure are ascribed primarily to the changes in the physical conditions of the system already noted and not to variation in the rate of CO_2 production of the tissues.

The tissues of the apples were brown on removal from the container, the cytoplasm was apparently disorganized and the cell sap had exuded into the intercellular spaces, making the tissues pulpy and moist.

In Experiment III, Fig. 2 c, a similar toxic effect was obtained in 5 atm. oxygen. At no time did the CO_2 output in the 'adjusted state' at 5 atm. oxygen reach the rate in the 'adjusted state' at 1 atm. oxygen, and after an initial apparent increase showed a steady falling off.

In Experiment IV the apples were put into the container with 1 atm. 100 per cent. oxygen and after the 'adjusted state' had been reached, the gas was replaced by 5 atm. 20 per cent. oxygen which gives the same partial pressure of oxygen. After the 'adjusted state' had been again reached the

pressure was reduced to 1 atm. 20 per cent. oxygen and then after a further interval was increased to 5 atm. 20 per cent. oxygen. Then the CO_2 output in the 'adjusted state' approximated to that for 100 per cent. oxygen in the

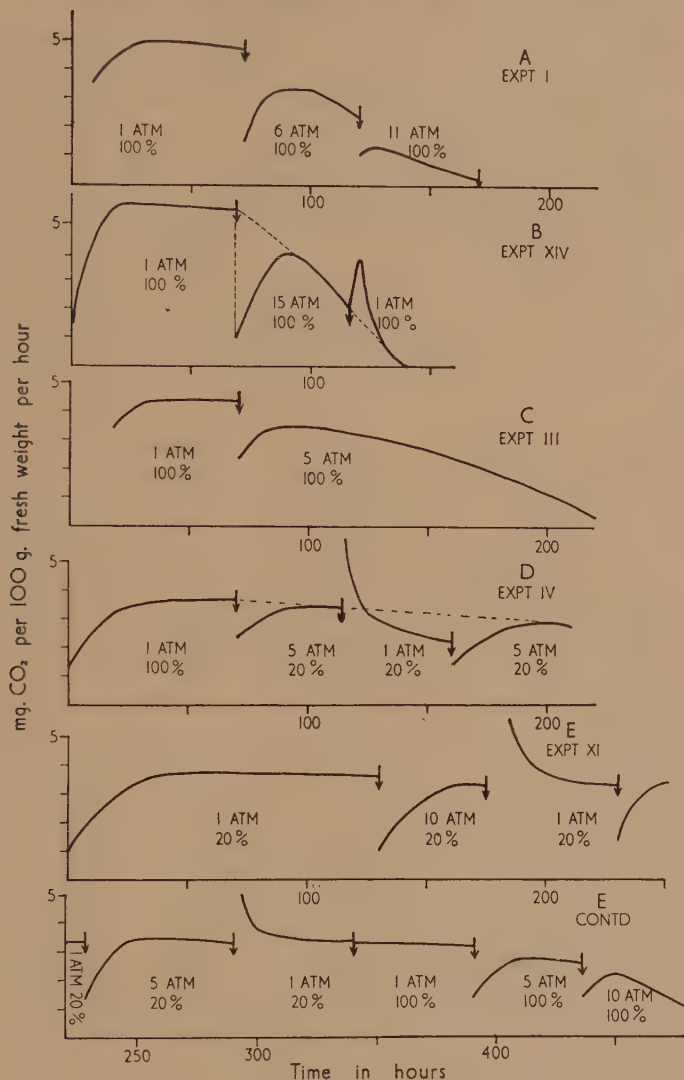


FIG. 2. Rates of carbon dioxide output (mg. 100 g. fresh wt. hour) by apples subjected to a variety of gas treatments indicated in the different curves. Time in hours shown as abscissae.

earlier stages of the experiment allowing for the expected fall in the output curve (see broken line in Fig. 2D). The apples were completely normal in appearance on removal from the container after a total period of experimentation of 210 hours. It would appear from these results that the tissues

were not damaged by *actual* pressures of 5 atmospheres and that the CO_2 output at 5 atm. 20 per cent. oxygen is approximately the same as that in 1 atm. 100 per cent. oxygen, i.e. in the same *partial* pressure of oxygen. Various other experiments confirmed this general conclusion.

It should be noted that in the latter part of their storage period, e.g. in May, some of the experimental apples showed signs of fungal infection after being kept in the container at 22.5°C . for some time. They had been stored at $+3^\circ \text{C}$. since harvesting in the previous autumn. In these cases there was a tendency for the CO_2 output to increase during the course of the experiment and some of the results obtained had to be treated with caution. In these cases the appearance of the fruit on removal from the container confirmed that fungal infection had taken place and due regard was paid to this fact.

There was clear evidence further that at the end of the senescent period the apple tissue was rather less responsive to the variation in the partial pressure of oxygen (at least up to 100 per cent. oxygen) than they were in the earlier stages of senescence. This point has been discussed by Blackman and Parija (1928).

Experiment XI was begun in 1 atm. 20 per cent. oxygen. After the 'adjusted state' had been reached, the pressure was raised to 10 atm. 20 per cent. oxygen then subsequently reduced again to 1 atm. 20 per cent. oxygen, again raised to 5 atm. and further reduced to 1 atm. It appears from the course of the curve of CO_2 output that 10 atm. 20 per cent. oxygen may have had a slightly depressant effect. After the periods in 1 atm. 20 per cent. oxygen and 1 atm. 100 per cent. oxygen, the pressure was raised to 5 atm. 100 per cent. oxygen and later to 10 atm. when the CO_2 output began as usual to fall steeply (see Fig. 2E). The tissues were, again, completely brown on removal from the container.

Experiment XIII also gave results which confirm that the CO_2 output is dependent on *partial* pressures rather than on actual pressures of oxygen. In this experiment the apples were put at 1 atm. 20 per cent. oxygen until the 'adjusted state' was reached. The pressure was then increased to 5 atm. 20 per cent. oxygen when there was a slight increase in the CO_2 output at the 'adjusted state' (see Fig. 3A). The pressure was again reduced to 1 atm. Thereafter 1 atm. oxygen was substituted until the 'adjusted state' was again reached, when 1 atm. 20 per cent. oxygen was again supplied followed by 1 atm. 100 per cent. oxygen and 15 atm. oxygen when the CO_2 output fell off rapidly.

In Experiment XV (Fig. 3B) high pressures of 2.5 per cent. oxygen were used and it was found that 16 atm. gave an appreciably higher output of CO_2 than 1 or 8 atm., and on returning to 1 atm. a level of CO_2 output was obtained which indicates that the tissues were not permanently or adversely affected by the treatments. The apples appeared normal on removal. In other words, the CO_2 output curve followed the line which would have been expected.

These effects were also shown in Experiment XX (see Fig. 3C) where the apples were put initially into 1 atm. 2.5 per cent. oxygen. The pressure was

then successively increased to 20 atm., reduced to 1 atm.; 1 atm. 20 per cent. oxygen was then substituted followed by 2.5 atm. 20 per cent. oxygen, 1 atm. 100 per cent. oxygen, 1 atm. 5 per cent. oxygen, 20 atm. and then

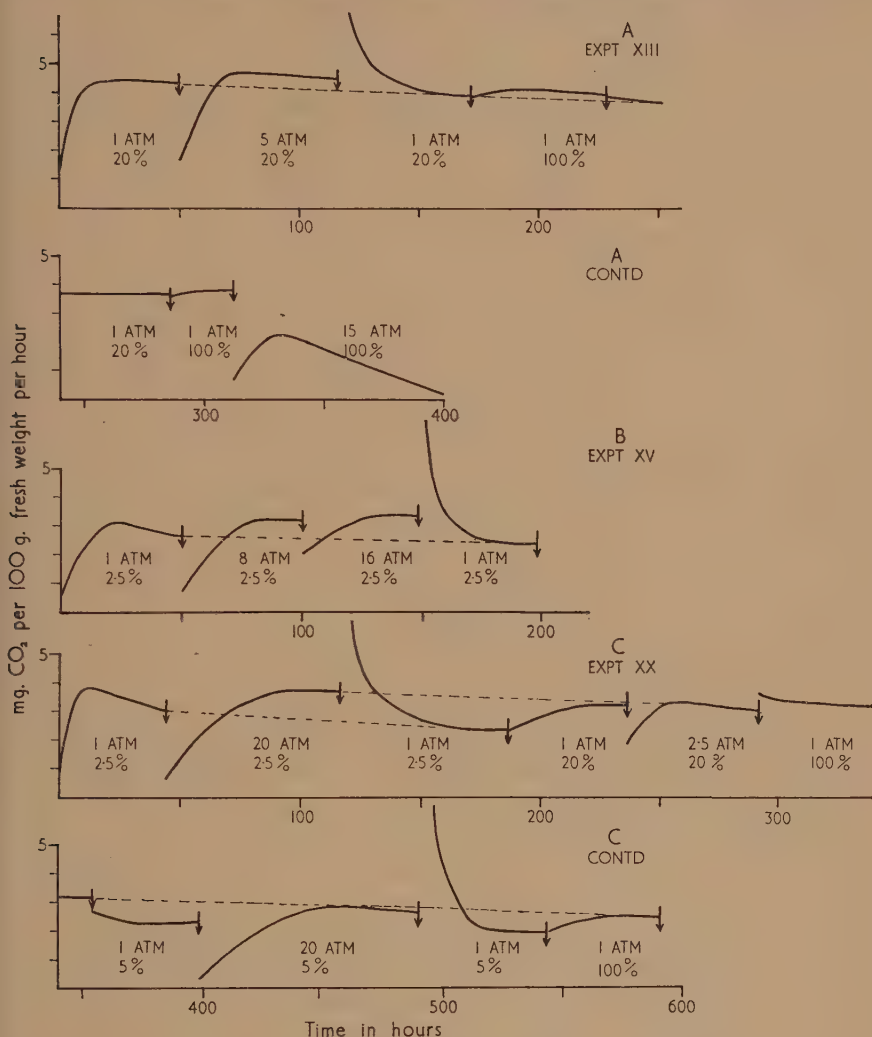


FIG. 3. Rates of carbon dioxide output (mg./100 g. fresh wt./hour) by apples subjected to a variety of gas treatments indicated in the different curves. Time in hours shown as abscissae.

1 atm. 5 per cent. oxygen; followed by 1 atm. 100 per cent. oxygen when the apples were removed and were found to be very slightly attacked by fungi.

DISCUSSION

The work of the members of the Cambridge School and others has shown that with variation in the oxygen concentration there is in general with apples

an increase in CO_2 output with increasing oxygen pressures up to 100 per cent. The investigation described in this paper relates in the main to pressures above atmospheric.

The toxic effect of high pressures of oxygen. Such evidence as is available from the whole series of experiments indicates that there was little significant increase of CO_2 output in 100 per cent. oxygen at pressures up to, say, 2 atm. It is possible that the increase was too small for accurate measurement. What was abundantly clear was that pressures of the order of 5 atm. oxygen caused a reduction of CO_2 output and were actually toxic within a comparatively short time. It will be noted that this lethal effect is associated with high *partial* pressures of oxygen and not with high *actual* pressures as such. The CO_2 output in 5 atm. 20 per cent. oxygen is substantially the same as that in 1 atm. 100 per cent. oxygen and that in 20 atm. 5 per cent. equal to that in 1 atm. 100 per cent. oxygen. Both 20 atm. 5 and 2.5 per cent. oxygen had no apparently deleterious effect on the apple tissues after exposure to these pressures for as long as 72 hours. This forces one to the conclusion that the toxic effect of high concentrations of oxygen is due to a specific effect of the oxygen itself and that these high concentrations of oxygen so interfere with the enzyme mechanism of the respiratory system that the cells of the tissues are ultimately killed. The breakdown of the protoplasts and the resulting browning and 'pulpiness' of the tissues are secondary effects following on the damage to this mechanism and the consequent death of the cells.

Support is lent to this view by observations following exposure of the tissues to high pressure of oxygen for short periods, e.g. in Experiment XIX 20 atm. for 6 hours. When the pressure was released and the apples examined after removal from the container, it was recorded that 'brown patches, unevenly distributed, were found in both apples. In the case of the larger, a uniformly yellow-green apple, the distribution was more regular. Each of the brown patches was associated with a lenticel. The other apple was red only on one side and it is of interest to note that it was on the red side that the brown areas were much more marked. . . . It was further observed that there was little tendency for the brown areas to spread. After some weeks the original brown areas dried out but had not in the interval increased in size except in those places where the skin was secondarily damaged' (Caldwell, 1931). The damage to the tissues following fungal infection did, on the other hand, spread rapidly. It is noteworthy that there was no evidence of secondary increase in CO_2 production as a result of fungal and/or bacterial infection of the disorganized tissues after high oxygen pressures.

A further consideration which suggests that the effects on CO_2 output was more closely associated with the concentration or *partial* pressure of oxygen than with the *actual* pressure of the gas is the period which is required for the attainment of the 'adjusted state' in CO_2 output in any given conditions. It will be noticed that, in the figures illustrating the experiments, this period is approximately 30 hours and remains more or less constant for all pressures where the oxygen concentration is not lethal. It would appear that the *actual*

pressure was of no greater and probably less importance than the *partial* pressure.

The data given in Fig. 4 (Expt. XVI) indicate that there was no marked increase initially in CO₂ output on exposure to high oxygen pressure. If the necessary extrapolations are made to enclose the areas *A* and *B*, after an exposure to 10 atm. 100 per cent. oxygen for only 24 hours and a subsequent reduction to 1 atm., the areas of *A* and *B* are found to be 65 and 58 units respectively.

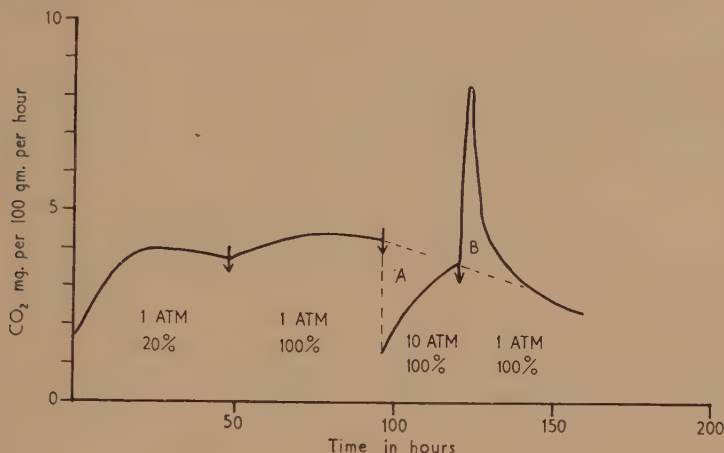


FIG. 4. Rate of carbon dioxide output of apple under high oxygen pressure.

Another conclusion which can be drawn from the above results is that, in general, the higher the oxygen pressure, the shorter the time required to kill the tissue. It is noted above that 6 hours' exposure to 20 atm. oxygen was sufficient to cause the death of at least groups of cells on the surface of the apple. See Table I.

TABLE I

Time required for cessation of CO₂ output under high pressures of oxygen

Pressure (atm.)	Time (hours)	Experiment
16	72	II
15	80	XIII
15	70	XIV
10	100	XII
9	100	VII
5	144	III

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Dr. J. Barker, F.R.S., for much valuable help in the preparation of this paper and to Mr. T. J. Wallace for his help with the graphs.

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The Translocation of Sulphonamides in Higher Plants

I. UPTAKE AND TRANSLOCATION IN BROAD BEANS

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SUMMARY

The uptake and translocation of sulphanilamide, sulphacetamide, sulphaguandinine, sulphapyridine, sulphadiazine, sulphathiazole, and 4:4-diaminodiphenylsulphone by broad bean plants growing in water culture has been studied. After varying times of exposure to the compounds at 100 µg./ml., total sulphonamide present in the roots, stems, and leaves was determined in acid-hydrolysed macerates by diazotization and coupling of the primary amino group. These compounds were identified in the leaves of treated plants by paper chromatography.

Accumulation of sulphonamide in the roots appears to be related simply to time, and the concentration of sulphonamide may, eventually, be far higher than that in the treating solution. Movement from the roots to the stems and leaves depends on transpiration. Sulphanilamide and the sulphone passed rapidly into the leaves; sulphacetamide, sulphapyridine, sulphadiazine, and sulphathiazole moved less rapidly. There was, however, a marked accumulation of sulphacetamide, sulphapyridine, sulphadiazine, sulphathiazole, and the sulphone in the roots. Sulphaguanidine was poorly absorbed from the treating solution.

INTRODUCTION

THE possible systemic movement of sulphonamides in plants was demonstrated by Hassebrauk (1938) who showed that soil treatment with *o*- and *p*-toluene-sulphonamide would markedly reduce rust disease on the leaves of wheat. These studies were later extended to include a wider range of sulphonamides and a more varied selection of rusts (Hassebrauk, 1951, 1952, *a, b*). It was then shown that sulphanilamide, sulphacetamide, sulphadiazine, sulphamerazine and 6(*p*-aminobenzenesulphonamido)-2:4-dimethylpyrimidine when applied to the roots were effective in controlling *Puccinia triticina* Erikss. on wheat, *P. simplex* (Körn) Erikss. and Henn. on barley, also *P. coronata* Corda on oats, and *P. dispersa* Erikss. and Henn. on rye. At various times these results have been confirmed by Hart and Allison (1939), Straib (1941), and Hotson (1951, 1953). Further indirect evidence of the translocation of sulphonamides in plants is provided by claims of the successful treatment of virus diseases, namely *X* disease of peach (Stoddard, 1947) and carnation mosaic (Thomas and Baker, 1949).

In the research so far reported it has been assumed that the sulphonamides act systemically, but no evidence of their systemic distribution in higher plants has been presented. Rudd Jones and Wignall (1955) have demonstrated the

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translocation of sulphanilamide and its acetylation in the tissues of the broad bean (*Vicia faba* L.) and the present report describes the translocation of seven sulphonamides in more detail.

MATERIALS

Six sulphonamides and one sulphone have been used in these trials:—sulphanilamide (*p*-aminobenzenesulphonamide), sulphacetamide (*p*-aminobenzenesulphonacetamide), sulphaguanidine (*p*-aminobenzenesulphonylguanidine), sulphapyridine (2-(*p*-aminobenzenesulphonamido)-pyridine), sulphadiazine (2-(*p*-aminobenzenesulphonamido)-pyrimidine), sulphathiazole (2-(*p*-aminobenzenesulphonamido)-thiazole) and 4:4'-diaminodiphenylsulphone which will be referred to simply as sulphone.

METHODS

In all trials the chemicals were applied to the roots of Sutton's dwarf variety of broad bean growing in water. The seeds were germinated in vermiculite. When the second leaf was beginning to expand, the seedlings were transferred to beakers containing nutrient solutions (Crowdy *et al.*, 1955). There were two plants in each beaker supported by formica disks on perspex cylinders. When the third leaf was fully expanded the nutrient was replaced with aqueous solutions containing 100 μ g./ml. of the sulphonamide being tested. Control plants were grown in demineralized water. The liquid level in the beakers was maintained by adding appropriate solution every three days and the solutions were changed completely each week. The water lost by each beaker during the experiment was determined by weighing each beaker at the beginning of the experiment, before and after each water change and at sampling. pH changes were followed throughout each experiment. Initially, the pH of the solutions of sulphanilamide, sulphacetamide, and sulphaguanidine was 5.8–6.0. Solutions of the first two compounds tended to become more acid after three days, reaching pH 5.0–5.5, whereas the sulphaguanidine became more alkaline, reaching 6.5. Sulphapyridine, sulphadiazine, and sulphathiazole had an initial pH 6.0–6.5 which fell to 5.0–5.5 after three days but subsequently rose again to the initial pH. The sulphone treatment was started at pH 6.5 and the acidity remained more or less constant until the plants were killed when it fell suddenly to 3.0–3.5. The pH of the demineralized water in control beakers was between 5.0–5.5 and it became more alkaline, reaching 6.0–6.5 after six days.

The sampling unit was one beaker with two plants and the plants when sampled were divided into roots, stems, and leaves. Four treated and two untreated units were sampled at each time. Before determining the sulphonamide, the tissues were weighed fresh and macerated in 0.5 N. hydrochloric acid. The final added volume was 50 ml. The acid macerates were boiled for 1 hour, made up to weight with distilled water and filtered. Acid hydrolysis released free sulphonamide from the N⁴ acetyl compounds. The estimate of sulphonamide was, therefore, of the total drug present and took no account of its form.

The amount of sulphonamide present in the filtrates was determined by the method developed by Bratton and Marshall (1939) as modified by Rose and Bevan (1944). Three ml. samples of the hydrolysed macerates were transferred to a 15 ml. centrifuge tube, neutralized with 2.5 N. sodium hydroxide; 2 ml. of 15 per cent. trichloroacetic acid were added to precipitate proteins; the tubes were shaken and centrifuged for 15 minutes at 935 R.C.F.; after centrifuging the sulphonamide was diazotized by the addition of 1 ml. of 0.1 per cent. sodium nitrite; the tubes were then shaken and 2 ml. of 1 per cent. N- β -sulphatoethyl-*m*-toluidine were added. A red colour developed after the addition of this coupling component. A second centrifuging for 5 minutes only gave clear solutions whose optical density was determined with a spectrophotometer at 510 m μ . The coloured compound formed with the sulphone tended to aggregate and it was necessary, therefore, to transfer the solutions to second tubes after protein precipitation and to resuspend the aggregate by shaking before determining the optical density in the spectrophotometer.

The colour of the plant sap did not interfere with the determination of the optical density of the diazo colour in neutral or slightly acid solutions, but alkaline hydrolysates were a dark red colour. In calculating the amount of sulphonamide present, the optical density of standard solutions of the drug of 50 and 100 μ g./ml. in control plant extract treated as above was plotted against concentration, and the standard line obtained was used to determine the concentration of sulphonamides present in the plant extracts. Agreement with Beer's Law was good up to 150 μ g./ml., but extracts containing more than 100 μ g./ml. were redetermined after dilution.

This method of determination is in fact an assay for amino groups in direct attachment to an aromatic nucleus and is not specific for a particular compound. However, paper chromatograms of plant sap developed in a butanol-ammonia-water system have shown that in the case of plants treated with sulphanilamide this compound was the only primary aromatic amine present in assayable quantities (Rudd Jones and Wignall, 1955). In the present trials the remaining sulphonamides have been similarly identified in sap expressed from the roots, stems, and leaves of treated plants.

EXPERIMENTAL RESULTS

There are two stages to be considered in a preliminary examination of the translocation of chemicals applied to the roots of plants, uptake by the roots and movement from the roots into the shoots. Previous work with griseofulvin (Crowdy *et al.*, 1955) has shown that uptake by the roots can also be separated into two processes, an initial rapid uptake, which is sensitive to sodium azide, and superimposed upon this a continuous uptake which is not sensitive to this respiratory inhibitor.

A comparison has been made, therefore, between the uptake of sulphanilamide by whole plants and by detached roots in an attempt to separate the uptake, and movement into the shoot, stages of translocation. Sodium azide

was also included in the experiment to distinguish between the two processes of uptake. Details of the experiment are illustrated in Tables I, II, and III, and Fig. 1. Whole plants and detached roots were treated overnight either in water or 10^{-5} M. sodium azide in water, a concentration which does not reduce transpiration (Crowdy *et al.*, 1955), and then transferred either to water or sodium azide solution containing $100 \mu\text{g./ml.}$ of sulphanilamide. Beakers, each containing two plants, were sampled in quadruplicate and the

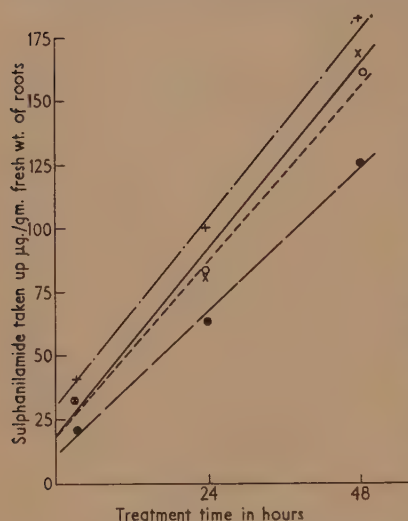


FIG. 1. Effect of sodium azide on the uptake of sulphanilamide; detached roots with azide \bullet — \bullet $y = 2.3t + 12.1$, detached roots without azide \times — \times $y = 3.04t + 19.1$, whole plants with azide \circ — \circ $y = 2.9t + 19.8$, whole plants without azide $+$ — $+$ $y = 3.13t + 29.7$.

sulphanilamide determined in hydrolysed macerates of roots and shoots at intervals up to 72 hours after the start of sulphanilamide treatment. At 72 hours the detached roots were senescent and this series of readings have been ignored. Fig. 1 illustrates the uptake of sulphanilamide by the roots of whole plants and by detached roots. The uptake by detached roots has been expressed as $\mu\text{g./g.}$ fresh weight of roots and for comparison the total sulphanilamide in the whole plants has also been expressed in the same units, $\mu\text{g./g.}$ fresh weight of roots; this measures the weight of the compound entering a unit weight of root and not the actual accumulation in the roots. The rate of uptake between 3.5 and 48 hours was constant and the same both for detached roots and for whole plants; it was unaffected by sodium azide. In Fig. 1 the best fitting lines are illustrated in each case, but within the limits of error all the lines have the same slope and can be

described by the equation:

$$y = 2.85t + a \quad (1)$$

where y is the weight of sulphanilamide taken up in time t . There was a significant difference in the position of these lines which indicated differences in the rate of uptake prior to 3.5 hours: a measure of these differences can be obtained from values of a in equation (1) which is, in effect, an extrapolation of the line to zero time. These values of a together with the calculated concentrations at 3.5 hours are shown in Table I.

Further information on the behaviour of the whole plants is presented in Tables II and III. The rate of accumulation in $\mu\text{g./g.}$ fresh weight of the tissue concerned was again found to be constant with time and the equations which show the accumulation are shown in Table II.

The rate of accumulation was significantly higher in the roots than in the shoots but it was not influenced by the presence or absence of azide. The

TABLE I

Sulphanilamide concentrations at zero time and 3.5 hours calculated from the equation $y = 2.85t + a$; expressed as $\mu\text{g./g.}$ fresh weight of roots

	0 hours	3.5 hours
Detached roots		
without azide	24	34
with azide	-1	9
Whole plants		
without azide	37	47
with azide	21	31

Differences significant at $P = 0.05$ exceed 16

TABLE II

Effect of sodium azide on the accumulation of sulphanilamide by the roots and shoots of broad beans. Concentration, y , in $\mu\text{g./g.}$ fresh weight accumulated in time, t , hours

Treatment	Individual equations	Combined regression coefficients $\mu\text{g./g.}$ fresh weight/hrs. units
Shoots		
without azide	$y = 1.02t + 6.4$	0.98
with azide	$y = 0.94t + 0.8$	
Roots		
without azide	$y = 1.44t + 26.37$	1.40
with azide	$y = 1.36t + 19.81$	

TABLE III

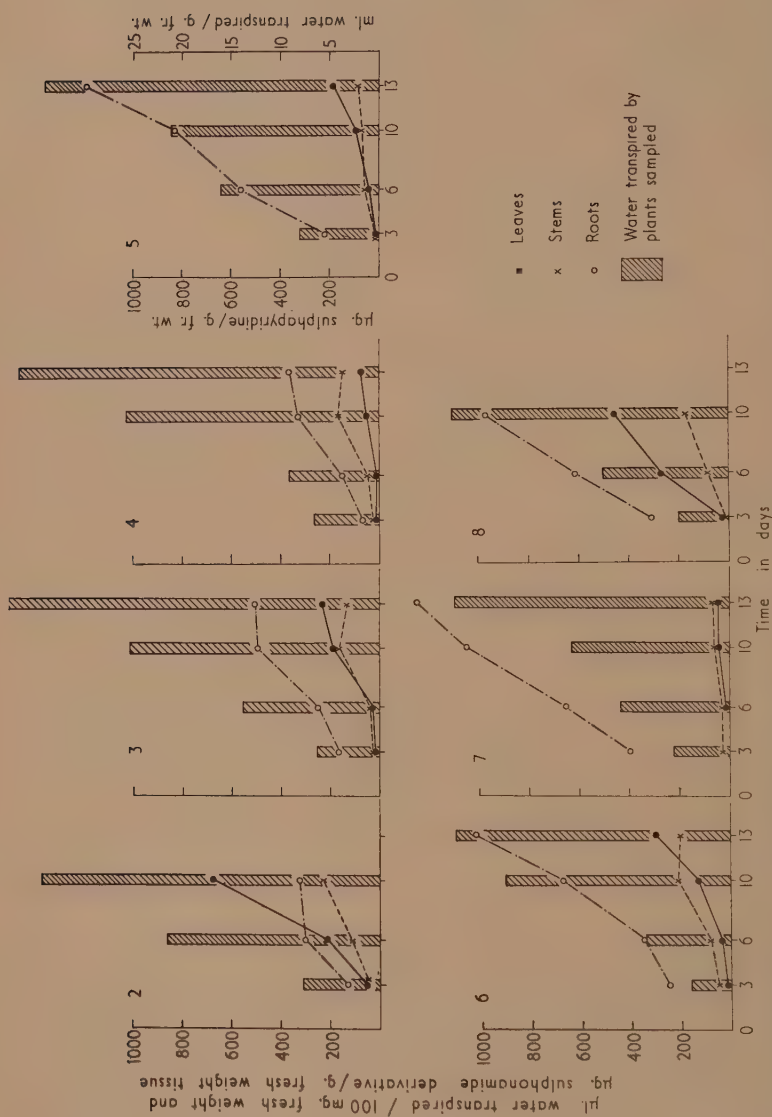
Sulphanilamide concentrations, $\mu\text{g./g.}$ fresh weight at zero time and 3.5 hours in shoots, calculated from the equation $y = 0.98t + a$, and in roots using the equation $y = 1.40t + a$

	0 hours	3.5 hours
Shoots		
without azide	7	11
with azide	-1	3
Roots		
without azide	27	32
with azide	18	23

Differences significant at $P = 0.05$ exceed 4.3 for shoots, 8.0 for roots; $P = 0.01$ exceed 6.2 for shoots, 11.2 for roots.

lines, however, differed significantly in position, indicating again differences in the rate of uptake prior to 3.5 hours associated with azide treatment: a measure of these differences is shown in Table III obtained by extrapolating the accumulation lines to zero time. This table also includes the calculated concentrations after 3.5 hours.

The results of experiments in which plants were treated with sulphonamides and sampled after 3, 6, 10, and 13 days are presented in Figs. 2 to 8. In these



FIGS. 2 to 8. Uptake of sulphonamide derivatives; leaves —•—; stems ---x---; rootso---; water transpired by plants sampled shown as shaded rectangles. FIG. 2. Sulphanilamide $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$. FIG. 3. Sulphacetamide $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NHCOCH}_3$. FIG. 4. Sulphaguanidine $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{N}=\text{C}(\text{NH}_2)_2$. FIG. 5. Sulphapyridine $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}(\text{C}_4\text{H}_3\text{N})$. FIG. 6. Sulphadiazine $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}(\text{C}_4\text{H}_3\text{N})$. FIG. 7. Sulphathiazole $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2(\text{C}_3\text{H}_2\text{NS})$. FIG. 8. Diaminodiphenylsulphone $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{C}_6\text{H}_4\text{NH}_2$. Ordinate units at extreme left refer to Figs. 2-4 and 6-8.

trials only one sulphonamide was included in each experiment and precise comparisons between chemicals are, therefore, impossible. However, the figures illustrate the general pattern of behaviour. In these experiments the transpiration, measured as ml. lost from the beakers per g. fresh weight of plant was a linear function of the treatment time for the untreated control plants, and a fall of transpiration rate of the treated plants was taken to indicate phytotoxicity of the compound under test. On this standard the plants treated with sulphanilamide showed phytotoxic damage in the 13-day sample though

TABLE IV

Rate of uptake of sulphanilamide and sulphacetamide as $\mu\text{g./g.}$ fresh weight per day and per ml./g. fresh weight (f.w.) water transpired

Experiment	Transpiration rate ml./μg. f.w.	Rate of sulphonamide uptake	
		μg./g. f.w. per day	μg./g. f.w. per ml./g. f.w. water uptake
Sulphanilamide A . .	1.0	32.3	30.9
B . .	1.5*	47.0†	30.1
Sulphacetamide A . .	1.4	33.9	23.5
B . .	2.2*	68.4†	28.6

* Difference significant at $P = 0.05$.

† " " = 0.01.

the symptoms were not obvious on inspection. The plants treated with sulphone were obviously wilted by the thirteenth day and no sample was taken; there was no reduction in transpiration by the tenth day. All the sulphonamides investigated accumulated in the leaves, stems, and roots of the treated plants. Sulphanilamide accumulated at approximately the same rate in the stems and roots and rather more quickly in the leaves. The remaining compounds accumulated preferentially in the roots and this accumulation was very marked with sulphadiazine, sulphathiazole, and the sulphone. The total uptake of sulphaguanidine was small and this compound was poorly mobile within the plant. The sulphone reached high concentrations in the leaves, whereas sulphathiazole only moved into the leaves very slowly.

From the single experiments for each compound described above, it is impossible to determine whether time or water uptake is the determining factor in the uptake of sulphonamide. However, further data are presented in Table IV which compares the results of two experiments with sulphanilamide and two with sulphacetamide in which uptake has been related both to time and to transpiration.

In both pairs of experiments the rate of transpiration was different and this difference was reflected in the rate of sulphonamide uptake when calculated on a time base. This difference between the experiments was eliminated when the rate of sulphonamide uptake was calculated as a function of transpiration. It appears, therefore, that transpiration is a major factor affecting the transport of sulphonamide, and the data would suggest that the experimental results

for the seven sulphonamides can be compared if allowance is made for differences in transpiration rate between experiments.

The relationship between transpiration and the concentration of the seven sulphonamides in whole plants is illustrated in Figs. 9 and 10. The data were derived from the experiments illustrated in Figs. 2–8 for the 3-, 6-, and 10-day samples. In the early stages (3- and 6-day samples) of sulphanilamide uptake when the concentration of compound in the tissues was low, the

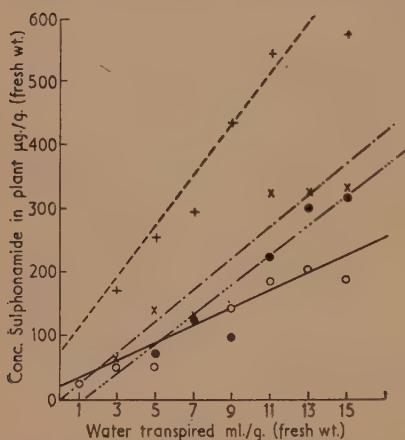


FIG. 9. Water uptake in ml./g. (fresh wt.) and concentration of sulphonamide in whole plant, $\mu\text{g./g. (fresh wt.)}$ — · · · · × sulphacetamide $y = 25.1x - 3.4$. — ○ sulphaguanidine $y = 14.1x + 17.6$. — · · · · ● sulphapyridine $y = 23.5x - 29.1$. — · · · · + sulphathiazole $y = 40.3x + 72.8$

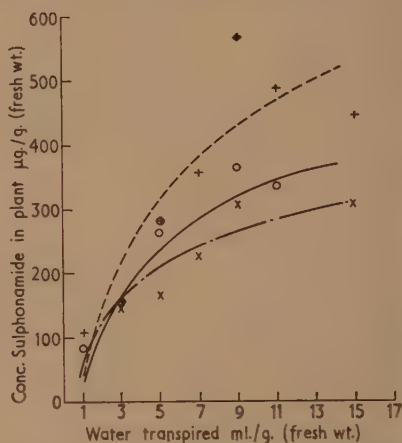


FIG. 10. Water uptake in ml./g. (fresh wt.) and concentration of sulphonamide in whole plant $\mu\text{g./g. (fresh wt.)}$ — · · · × sulphanilamide $y = 208 \log_{10} x + 65$ — ○ sulphadiazine $y = 307 \log_{10} x + 24$ — · · · + sulphone $y = 434 \log_{10} x + 15$.

concentration in the plant was a linear function of the water transpired; this relationship also held good for sulphacetamide, sulphaguanidine, sulphapyridine, and sulphathiazole for up to 10 days (Fig. 9). On the other hand, over the 10-day period, the rate at which the sulphone increased in the tissues was found to be a logarithmic function of the amount of water transpired. Sulphanilamide and sulphadiazine could be fitted by a linear or a logarithmic function, but the latter gave the better fit in both cases (Fig. 10). Whether the steady reduction in the rate of uptake of sulphanilamide, sulphadiazine, and the sulphone should be attributed to saturation of the tissues with these compounds, breakdown in the plant, or phytotoxicity, is not yet known.

To allow for comparison of the behaviour of the various sulphonamides tested their rates of accumulation were calculated as linear regressions of total $\mu\text{g.}$ recovered against total water transpired over the linear phase of uptake. The accumulation rates for leaves, stems, and roots for the seven compounds are shown in Table V. The differences in the table relate strictly to differences between experiments and may include factors other than chemical treatment.

However, since the presentation of the data eliminates the effect of transpiration, and since there was good agreement between consecutive experiments with one sulphonamide, it was felt that the residual effects could fairly be attributed to differences in the behaviour of the compounds tested.

The rates of accumulation were lower for stems than for leaves or roots for all sulphonamides except sulphaguanidine and sulphathiazole and with these two compounds movement through the plants was very slow. With sulphanilamide and the sulphone the rates of accumulation in the leaves were very high, whilst with the other sulphonamides a low rate of accumulation in the leaves was associated with a high rate in the roots and this was particularly marked

TABLE V

Rates of accumulation of sulphonamides in the tissues of broad beans in $\mu\text{g./ml.}$ water transpired, calculated from linear regressions

	Leaves	Stems	Roots	Whole plants	Shoots
Sulphone	30.3	8.0	20.2	58.5	38.3
Sulphathiazole	0.6	1.0	38.7	40.3	1.6
Sulphadiazine	7.1	7.7	20.0	34.8	14.8
Sulphanilamide	19.7	3.0	8.5	31.2	22.7
Sulphacetamide	7.8	2.2	15.1	25.1	10.0
Sulphapyridine	1.3	0.8	21.4	23.5	2.1
Sulphaguanidine	2.0	3.5	8.6	14.1	5.5

in the N^1 heterocyclic-ring substituted sulphonamides, sulphapyridine, sulphadiazine, and sulphathiazole. The sulphone also showed marked accumulation in the roots.

It must be remembered when considering Table V that two processes are involved. The first is entry into the plant for which the source of supply is a practically inexhaustible reservoir of $100 \mu\text{g./ml.}$ solution surrounding the roots. The second is movement into the shoots in which case the source of supply is the compound present in the roots, and in this case the amount of sulphonamide available for translocation will be determined by its rate of entry from the surrounding solution and its retention in the root tissues. Similarly the movement into the leaves will be conditioned by the sulphonamide in the stem. The data from Table V have been presented in Table VI as the sulphonamide actually translocated as a percentage of that available for translocation in each part. The percentages for sulphanilamide were calculated as follows: from a treating solution of $100 \mu\text{g./ml.}$, $31 \mu\text{g./ml.}$ enter the plant, of this $23 \mu\text{g./ml.}$, or 73 per cent., leave the roots for the shoots; of the $23 \mu\text{g./ml.}$ entering the stems $20 \mu\text{g./ml.}$, or 87 per cent. are translocated to the leaves.

In all cases, the apparent concentration of a compound in the water stream entering the plant is considerably less than the concentration in the solution surrounding the roots. The highest concentration was in the plants treated with sulphone in which the concentration of the entering solution was 59 per

cent. of the surrounding solution. Sulphathiazole, sulphadiazine, and sulphanilamide form an intermediate group with 30–40 per cent. entering, sulphacetamide and sulphapyridine enter at about 25 per cent., whilst the entry of sulphaguanidine is very low. Some measure of the amount available for translocation will be given by the proportion of available compound translocated from the roots and stems. The sulphone and sulphanilamide are readily available, sulphadiazine and sulphaguanidine less so, whilst sulphathiazole and sulphapyridine are strongly retained. There is little retention of the

TABLE VI

Percentages of available sulphonamide translocated from the tissues of broad bean

Compound	Percentage of sulphonamide translocated		
	From solution to plants	From roots to shoots	From stems to leaves
Sulphone	59	66	79
Sulphathiazole	40	4	38
Sulphadiazine	35	43	48
Sulphanilamide	31	73	87
Sulphacetamide	25	40	78
Sulphapyridine	24	9	62
Sulphaguanidine	14	39	36

sulphonamides in the stem, since even with sulphathiazole 38 per cent. is translocated from stems to leaves as compared with only 4 per cent. entering the stem from the roots.

DISCUSSION

These experiments have shown that a number of sulphonamides are taken in by the roots of broad beans and translocated either unchanged or in a form from which the sulphonamide may be released readily by acid hydrolysis.

The data from the 72 hours' experiment indicated a steady intake of sulphanilamide over the period. The rate of uptake from the solution was dependent on the size of the root system, since the sulphanilamide entering per unit weight of roots was the same for roots of whole plants and for detached roots. In detached roots the total intake was accumulated while in whole plants accumulation was divided between the roots and shoots, the rate of accumulation being constant in each case and significantly higher in the roots. This steady uptake was not affected by azide at any stage. Superimposed on this steady uptake with time was a rapid uptake which was completed before the first reading at 3.5 hours. This also appeared to be made up of two effects: one which was sensitive to azide and which accounted for all the initial rapid uptake by the detached roots (Table I) and the initial rapid entry into the shoots (Table III), and a second which was not affected by azide and which occurred only in the roots of whole plants. This is shown in Tables I and II as a significant initial uptake by the roots of azide-treated whole plants.

Over longer periods of time the evidence suggests that sulphonamide uptake

is conditioned by the rate of transpiration. This effect has also been recorded for griseofulvin (Crowdy *et al.*, 1956). This would be expected since once a steady state has been achieved between the sulphonamide in the roots and that in the surrounding solution further entry into the roots will balance removal of the compounds from the roots and the most likely path of removal will be the transpiration stream. The rate of movement in the transpiration stream will be controlled by a complex of factors including the solubility of the compound, or its N^4 acetyl derivative should it be readily acetylated, and adsorption in the tissues.

In the long-term experiments the sulphonamide in the roots reached concentrations far in excess of those in the surrounding solution. It should be noted, however, that the main part of this build-up was not azide sensitive and there was no real evidence that the concentration of sulphonamide free in the aqueous phase in the tissue was higher than in the surrounding solution; the compound may be sequestered as the N^4 acetyl derivative or by some physical binding. There is, therefore, no evidence of an active phase of uptake. The uptake behaviour and its reaction to azide treatment is similar in many respects to that reported for griseofulvin (Crowdy *et al.*, 1956) and the present experiments throw no light on the nature of the processes involved.

ACKNOWLEDGEMENTS

We are most grateful to Miss H. Morgan, Miss A. Petrie, Miss K. C. Robinson, Mr. B. Langford, and Mr. P. Richmond for technical assistance.

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Studies in Extension Growth

I. A NEW CONTACT AUXANOMETER

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Received 11 November 1955

SUMMARY

Various types of auxanometer are discussed, and a new contact auxanometer is described that is capable of recording a total extension of about 0.4 cm. to within an accuracy of 0.5μ per minute. Some specimen records of the extension of etiolated *Vicia faba* epicotyls showing some effects of mechanical stimulation are given.

INTRODUCTION

ALTHOUGH the use of simple auxanometers for the demonstration of the progress of growth by means of the increase in length of plant organs is very old, auxanometers cannot be said to have become in any way standard physiological instruments. In the past the great investigations into the light-growth reactions of coleoptiles yielded but one machine designed to record these phenomena. The bulk of the work was performed by patient observation by microscope of the plant itself, often in difficult conditions of illumination.

During the last decade or so the techniques of bio-assay have developed in such a way that a very accurate knowledge of the extension rate of plant parts is required; but while the conditions of culture of the material have been greatly refined, the actual measurements are still of the crudest type. Investigations into the extension growth of the stems of *Vicia faba* seedlings, of which this forms the first part, have shown that the extension rate of individual plants is extremely sensitive to stimulation or environmental change, and that the fluctuations in the rate are rational. It is believed that records of the rate of elongation of plant parts, if of sufficient accuracy and duration, may be valuable as a means of detecting and observing characteristics of extension processes that have hitherto been unsuspected or ignored.

The magnification that may be attempted with the simple Ganong-type auxanometer is limited by the time available before resetting is required. At large magnification a coupled lever type of instrument will only be suitable for short investigations. This is one of the limitations of the 'Crescograph' of Bosc (1927).

It is also true that as the magnification of this type of instrument is increased, the range of reading that may be expected for a repeated application of the same initial displacement is also increased. This is due to loss of energy

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at the couplings and is found in all but optical instruments; it constitutes the variance of the instrument. Another property of instruments that is of importance to auxanometer design is what may be termed the passiveness of the mechanism, and is manifest as the existence of a definite smallest alteration of the observed value required to move the indicator at all. This clearly limits the sensitivity of the instrument. The effect is associated with the amount of work that has to be done to move the mechanism. Moreover, when the plant is the prime controller, this energy is intermittently released by reason of the elasticity of the tissue, since the growth processes are reacting against a temporarily rigid body. Since plant tissue is weak and very sensitive to mechanical deformation, it is important that the passiveness of the auxanometer is small. It is evident that all instruments of this type, i.e. that derive all or most of the energy for their action from the plant itself, have serious limitations. The Interferometer Auxanometer of Meissner (1932), while being the most sensitive instrument both in the literature and in theory, retains these defects.

Another class of instruments includes those that are fed with energy in order to control some value, such as thermostats and voltage regulators. The 'Balanced Crescograph' of Bose was of this type. In this machine the plant was attached to a lever system of high ratio, but was moved in the direction opposite to its elongation by a clockwork mechanism of variable speed, which was initially adjusted so that the levers did not move. Any deviation from the initial rate of extension was then indicated by a departure from the null position of the levers. It will be seen that a self-regulating machine is possible that continually compares the speed of elongation of the plant with that of a part of the apparatus, converting any discrepancy in the two rates into a signal that alters the speed of the apparatus in the direction of equalization. The speed of the apparatus may be measured and recorded by methods not applicable to the plant itself.

The term 'Contact Auxanometer' refers to those automatic electric machines which use a switch as the position sensitive device. The discontinuity of the switch output is usually maintained throughout the system so that the mechanism expands in a series of discrete increments of known and constant size, and these are recorded as unit events in time. Many other types of instrument are possible if a position sensitive device with a continuous output is used, but none such appear to have been described.

Contact Auxanometers have been described by Bovie (1912), Koningsberger (1922), Hunter and Rich (1923), and Ranson and Harrison (1955), whose designs provided measurements of growth increments of respectively $25\ \mu$, $10\ \mu$, $5\ \mu$ (hand operated), and $22\ \mu$. One great advantage of the Contact Auxanometer is that the recording gear may be remote from the plant, since the only connexions are electrical. But if a direct mechanical connexion is convenient, then simpler apparatus of the type described by Idle (1955) may be used.

Possibly one of the most potent factors affecting auxanometer design is the

need for accommodating growth of variable sign. The standard contact auxanometer will not do this, and will give an inaccurate record if a change in direction occurs during an experiment. Moreover, in order to resolve the growth rate into periods of 10 minutes or less, increments should be smaller than 5μ . This necessitates a specialized advancing mechanism and switch, and also demands a recording technique adequate to deal with the information produced, for measurement of an active tissue may give tens of increments every minute. There is also the difficulty that should the switch fail to open after the mechanism has moved it, then the machine is jammed. This fault becomes limiting at increments of 5μ and under. In the following apparatus most of these difficulties have been overcome.

THE NEW INSTRUMENT

(i) Design

The auxanometer was required to measure the growth rate of the etiolated plumule of *Vicia faba* L. during the light-growth response, when the rate may vary from $10\text{--}20\mu/\text{min.}$ to nearly zero. Preliminary work had shown that the effects of illumination lasted for several hours, but the immediate reaction was not known. The basic time interval chosen for the new study was one minute. The apparatus to be described will record growth of from 0 to $80\mu/\text{min.}$ using 0.5μ increments, over a total extension of between 4 and 5 mm.

(a) *The advancing mechanism.* A Cambridge Rocking Microtome was stood on end supported by the knife holders, as shown in face view in Fig. 1. The ratchets and a solenoid *S* were arranged such that its action was to rotate the toothed wheel of the microtome and so raise the cutting head *C* away from the knife holders, which held the plant support *A*. The maximum travel was found to be about 0.5 cm. in a circle of such radius that for practical purposes the motion the cutting-head displayed was linear and vertical. The arrangement of teeth on the wheel made a choice of increment size possible to a minimum of 0.5μ .

(b) *The switch.* The best design found so far for this member is shown in Fig. 2. The contacts *C* are crossed platinum cylinders formed from 40 s.w.g. drawn wire threaded through the perspex mounts. The counterweights *W* ensure that there is very little pressure on the plant, and being pivoted instead of sprung this pressure changes little over the whole arc of motion. The contacts are normally open, upward movement of the lower contact causing closure.

(c) *The relay.* This is a conventional circuit (see Fig. 3) consisting of a triode which normally conducts but whose anode current is reduced to near cut-off by application of grid bias when the auxanometer switch is closed. The relay *R*₁ in the anode circuit is arranged so that the master contacts (*A*) that control the advancing and recording gear close when the anode current falls.

(d) *The recording mechanism.* The arrangement is shown in Fig. 4. The impulse motor *M*₃ moves the paper *P* about $\frac{1}{16}$ in. for each increment

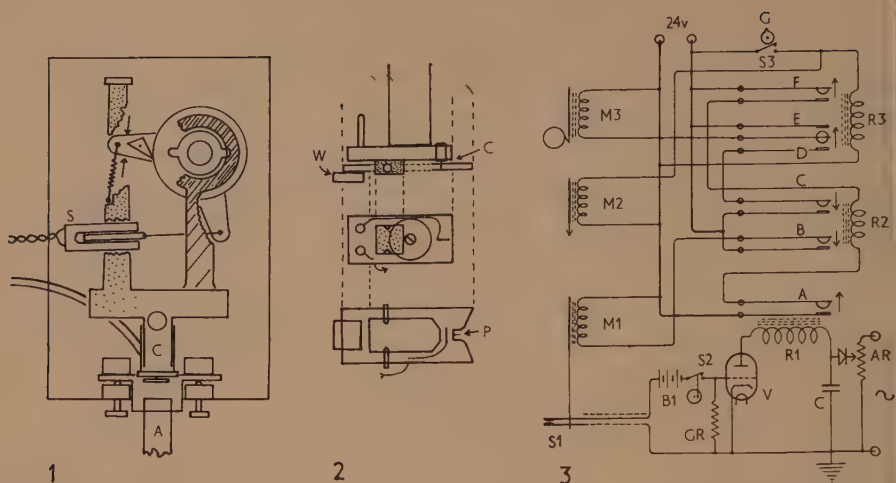


FIG. 1. Cut-away view of rocking microtome modified to form the advancing mechanism of the auxanometer. S, solenoid; C, cutting head of microtome bearing the switch; A, support for the plant. FIG. 2. Detailed construction of the switch. C, the contacts, of crossed platinum wire; W, counterweights; P, tungsten pin 0.5 mm. long. FIG. 3. Circuit of the relay. For operation see text. V. EC92; B1. 3v; GR. 5 Mohms; C. 2 mfd; R1. 10,000 ohms; AR. 20,000 ohms; AR. is supplied with 230 v. AC.

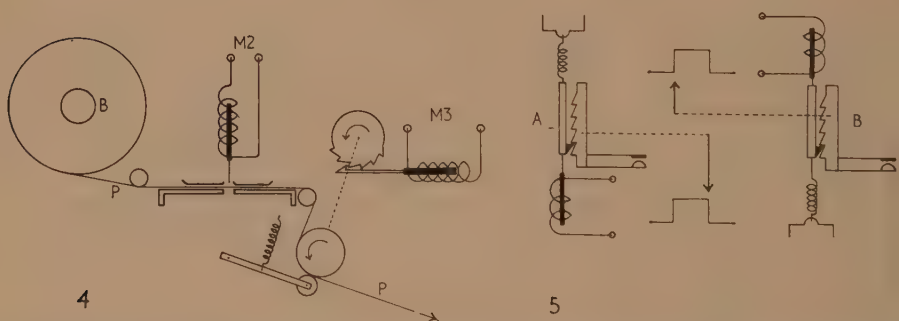


FIG. 4. The recording apparatus. See text. M3 advances the paper P; M2 punches the paper strip. FIG. 5. Two forms of advancing mechanism. A, the switch rises at the end of the energizing pulse; B, the switch rises when the voltage is applied to the system.

advanced by the auxanometer. The solenoid *M*₂ punches a hole in the strip with a needle at one-minute intervals. The strip is supplied from the reel *B* on the left and runs into a receptacle on the right. Immediately after the paper is punched by *M*₂, it is automatically moved on a distance equivalent to one increment. If this were not done then in conditions of no growth the holes would be coincident and it would be impossible to record elapsed time. This phantom increment is taken into account when the results are analysed. At the high rate of action in this apparatus, due to the small size of the increment this method of recording offers considerable advantages over the usual technique in ease of interpretation and economy of recording paper.

(e) *The overall action.* In Fig. 3 the auxanometer switch SI is in series with the battery BI and the switch $S2$, which is normally closed. When the plant closes SI , the valve is biased almost to cut-off, and the contacts A of the master relay $R1$ close. Since the contacts F of relay $R3$ are normally closed, completion of the circuit through A energizes the slave relay $R2$, which operates the heavy duty contacts B and C . The closure of B operates MI , which is the advancing mechanism of the microtome. The switch SI opens on being raised, allowing the valve to conduct and to re-energize $R1$. Contacts A then reopen, and B and C follow. The apparatus is then ready to repeat the cycle when the plant next closes SI .

The recording mechanism consists of the impulse motor $M3$ and the solenoid $M2$ that punches the paper strip. Since the contacts D are normally closed, $M3$ is operated by closure of the contacts C on the slave relay $R2$, and thus advances the paper each time the auxanometer cycle described above is completed. The timing of the action of $M2$, the solenoid that marks the paper every minute, depends upon the synchronous electric motor G , which closes the switch $S3$ once per minute. $S3$ also controls the relay $R3$, which has a double function. Operation of $R3$ closes the contacts E which cause $M3$ to advance the paper strip when it is punched. At the same time the contacts F open, preventing any action of $R2$, and hence the auxanometer, that might occur during the punching cycle. Thus any incipient action of MI is held over until it may be properly recorded.

As has been mentioned, it is a fault of contact auxanometers that if the switch fails to open then no further action can occur, and the machine is jammed unless the mechanism is such that it continues to advance until the switch clears itself. At small increments this fault renders the machine almost useless, as the failure becomes frequent. In Fig. 3 the switch $S2$ is of importance. It may be called the de-jamming switch, and it opens every $\frac{1}{3}$ second, so that if SI remains closed, a series of impulses are applied by $R1$ and $R2$ to MI , which advances repeatedly until SI reopens and resumes control.

A very potent cause of auxanometer switch wear and failure is the arc that may form across the minute gap between the contacts if there exists a potential across them at the moment of their separation. Such an arc will not only corrode the contacts, but will cause erratic movements of the relays, leading to inaccurate records. It has been found that to limit the current in the grid circuit to less than one microamp is not enough. By suitable design it has been possible to remove the responsibility for breaking the grid current from SI to $S2$, which may be properly designed to withstand corrosion.

In Fig. 5 A and B represent two forms of electromagnetic advancing mechanisms. Both have ratchets that raise a switch when the magnets are energized by a pulse of electricity, but it will be seen that they differ in the moment when the switch actually moves upwards; in A this happens at the end of the pulse when the electromagnet relaxes. In B the magnet pulls the switch up when the current begins to flow. The advancing mechanism of the microtome takes the A form, and therefore (Fig. 3) on closure of SI

by the plant and subsequent movement of M_1 , the switch S_1 is not opened by upward motion until the current is broken by the next opening of S_2 . Thus although the auxanometer switch is responsible for the initiation of an increment cycle, it is the de-jamming switch that ends it. In this form all electrical wear of S_1 is avoided. There is an additional advantage in that since each cycle of events includes a variable time-lag while S_2 comes round to its open position, the action of the advancing and recording mechanisms is more certain.

The maximum rate at which the apparatus can operate is limited by S_2 , which in this case opens three times every second. It is driven by a small synchronous electric motor.

The apparatus has always been used at 0.5μ increments, and the maximum rate is therefore $0.5 \times 180 = 90 \mu/\text{min}$.

(ii) Performance

(a) *Relay and recording mechanism.* It is possible to supply impulses to the relay at a constant rate by means of a pendulum-actuated switch in lieu of S_1 . This tests the reproducibility of minute-by-minute recording at sub-maximal rates. This experiment has been performed with the results shown in Fig. 6. Two lengths of pendulum were used to give inputs of 40 and of 20 'increments' per minute. The resultant graph of rate plotted against time, using measurements directly from the paper strip, ought to be a straight horizontal line. Periodic errors ought also to show under these conditions. There is in fact a suggestion at 20 impulses per minute of a periodic artefact, but it is within the degree of scatter liable to be produced at all speeds greater than $5 \mu/\text{min}$, i.e. 10 impulses per minute. When 10-minute averages are required, this scatter is not of importance, but if the record is to be studied for significant minute-by-minute changes of growth rate then this limitation must be taken into account.

This type of experiment may be performed with variable input by manual operation of S_1 . By connecting an electromagnetic counter in parallel with M_1 or M_3 it is possible to compare the record on the strip with the numbers taken from the counter each minute. The distances between the time intervals ought then to be proportional to the number of impulses shown to have been generated in those minutes. The results (bottom record of Fig. 6) corroborate the findings of the previous experiment with constant input.

(b) *Advancing mechanism and overall response.* The displacement per tooth was found to range from 0.45μ at one end of the screw, through a maximum of 0.5μ to 0.48μ at the other end. The displacement per tooth has been taken as 0.5μ when calculating the rate of growth.

It has not been possible to check the accuracy with which successive increments are produced by the microtome. It could be done by interferometry. It has been found to be difficult to check even groups of increments as produced under experimental conditions. Systems involving a micrometer slide fixed to the cutting head and followed by a microscope, or a clock-driven

screw in place of the plant, have proved to be less reliable with the equipment available than the machine under test. It is possible, however, to gain some idea of the departures from a smooth growth curve produced by the apparatus as a whole. A bar of metal may be expanded by the action of heat, and the

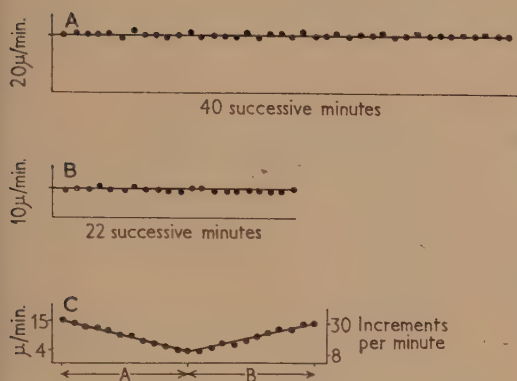


FIG. 6. The performance of the relay and recording mechanisms. Constant input: A, 40 impulses per minute applied to S_1 (Fig. 4); B, 20 impulses per minute applied to S_1 . Variable input: C, see text. Manual operation of S_1 . Period A, in decreasing, and B, increasing steps of two impulses each minute.

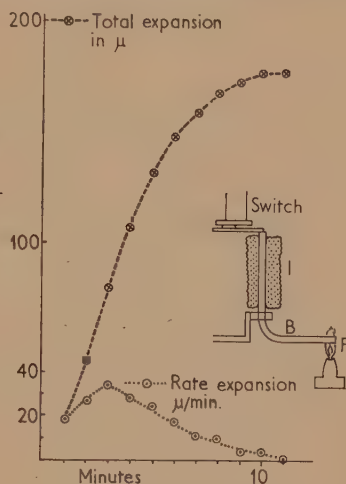


FIG. 7. Performance of the entire instrument. The apparatus is made to record the thermal expansion of a bar of aluminium B when heated at one end by a flame F, and insulated by the sponge I. The upper curve shows the total extension of the metal with time, and the lower curve shows this extension as rate with time. See text.

resulting elongation measured by the auxanometer. If the rate of supply of heat is constant or nearly so it is reasonable to assume that the rate of expansion will alter with time in a regular fashion, so that the record ought to be a smooth curve of some shape determined by the configuration used. But it is only possible to run such an experiment for a short while. Fig. 7 shows the results gained by this method. The errors do not appear to exceed those that arise during tests of the recording apparatus alone.

(c) *Long-term stability.* The auxanometer has run over 2,000 hours in periods of up to one week's continuous working without breakdown. But routine inspection and maintenance are necessary, and the components are of high quality.

(iii) Accessories

A pair of contacts were fitted to the microtome such that the approach of the nut to the end of the screw caused their closure. This provided a signal to switch off the apparatus automatically if so required.

In order to make it possible to gauge how much longer the apparatus would run during an experiment before requiring resetting, a counter was fitted in parallel with MI , so giving an indication of the total number of increments up to the time of observation. The maximum number was found to be of the order of 8,500, equivalent to $4,250\ \mu$, or 0.4 cm. approximately.

EXPERIMENTAL RESULTS

It is appropriate first to illustrate the applications of a sensitive auxanometer to growth studies with a description of the behaviour of etiolated broad bean seedlings extending in darkness.

(i) *Materials.* *Vicia faba* var. Mammoth Green Longpod seeds were soaked in water in darkness for 24 hours, and planted in perspex jars lined with filter-paper, so that each seed was held between the paper and the walls of the jar. There were six seeds to each jar, and the jars contained water to a depth sufficient to supply water to the plants for a period of 2 weeks. The jars were placed in a totally dark chamber, which was maintained at a temperature of $22.0^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$ by means of an energy regulator, so that there were no periodic fluctuations due to a control cycle. The humidity was maintained between 80 and 90 per cent. rh. The seeds germinated undisturbed, and were ready for use after about 11 days. They then had produced a plumule some 5 in. long, consisting entirely of the first internode, surmounted by a plumular hook region showing the first node. All experiments refer to the first internode of the seedling. This particular culture technique enables plants to be removed from the jars one at a time with the minimum injury to shoot or root, since the roots spread over the walls of the vessel, and do not penetrate the paper.

(ii) *Methods of mounting the plant.* Three methods successful for *Vicia faba* are shown in Fig. 8. The 'seed clip' method involves least damage to the stem of the plant. Using the 'stem clip' mount it is possible to choose more accurately the part of the stem it is wished to observe, down to segments only 0.5 cm. long. This has the desirable effect of not only extending the total time available for observation, by reason of the reduced extension rate, but also of selecting tissue composed of cells all of more or less equal maturity, so that the nature of events dependent in character upon age will appear more clearly defined in the record. The 'stem clip' method also enables stems cut from the remainder of the plant to be observed while supplied with fluid nutrients at the cut end.

The 'Kinematic' mount has not been used to any great extent, but it is probably a type suited to smaller and less robust plants than the bean. A more correct design would show the rubber supports to be V-shaped. All three methods have been used in complete darkness, but the mounting operation is then difficult, so that even with the simple seed clip it is preferable to use a light 'safe' for the particular study, in order to reduce the incidence of experimental failure.

(iii) *Experimental conditions.* The microtome unit of the auxanometer stood on a plinth in an incubator in a dark room. The room was controlled at

$20^{\circ}\text{C.} \pm 1.5^{\circ}\text{C.}$, and the incubator maintained at $23^{\circ}\text{C.} \pm 0.05^{\circ}\text{C.}$ by means of an internal fan and heating unit. This incubator control cycled at less than a minute, and used a bimetal strip of small dimensions as the sensing element.

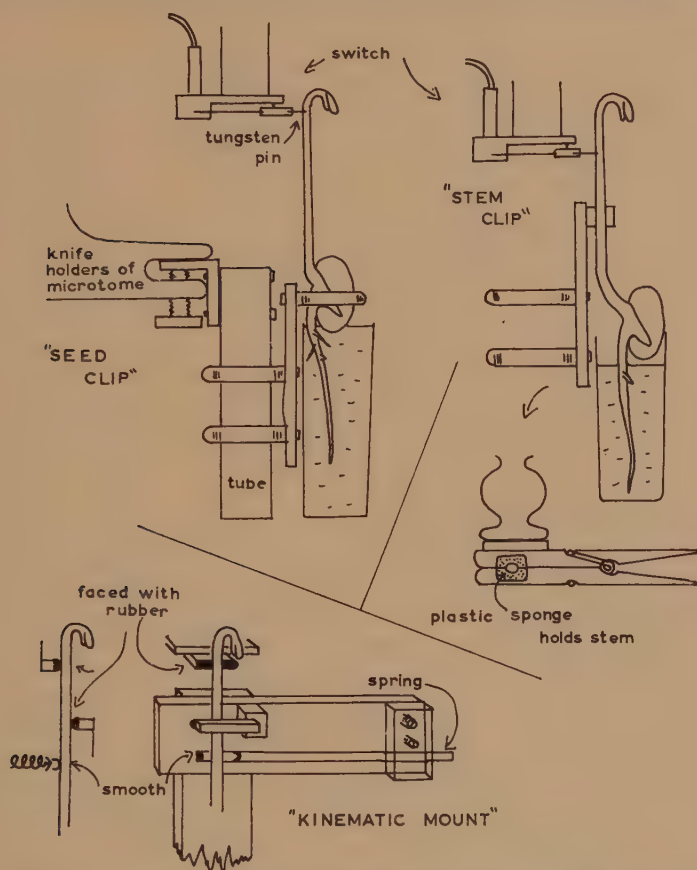


FIG. 8. Three methods of mounting the plant material for observation in the auxanometer.

The temperature cycle quoted above was measured by Beckmann thermometer, but owing to its large heat capacity it could not be expected to indicate the true extremes of the air temperature. However, these would not have exceeded about $\pm 0.1^{\circ}\text{C.}$ The incubator was light tight and the relative humidity maintained at about 95 per cent. by slow evaporation from a large surface inside the apparatus. The incubator was situated next to the germination chamber, so that the plants did not have to be carried far at the beginning of each experiment. During transfer and mounting a green light (Ilford safelight G 907 or 'Wratten' gelatine filter No. 54) was used and since the eye is most sensitive to green light, it may be used at a lower intensity than any other colour.

(iv) *Preliminary investigations.* Long records from some 50 plants were taken during the preliminary work. It was found that the extension rate fluctuated a great deal over periods of a few minutes, in no very regular fashion, and that the responses due to stimuli such as light and mechanical shock lasted a

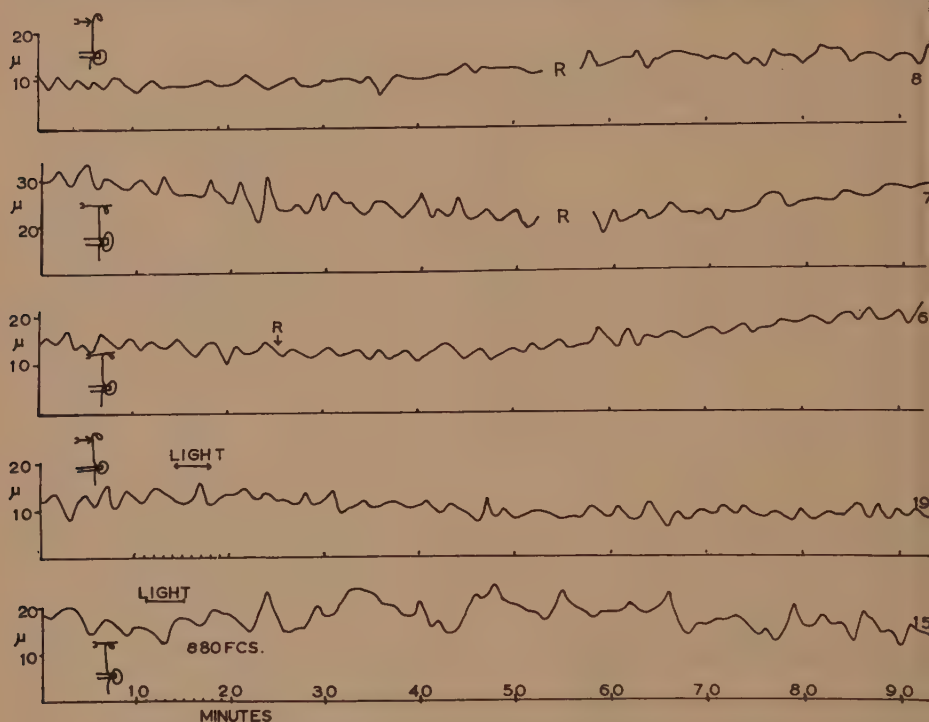


FIG. 9. Short-term observations of the extension rate of dark grown *Vicia faba* plumules. The graphs are plotted at the maximum resolution possible with the apparatus, namely one point each minute. The insets indicate the type of switch used (see text). R indicates that the apparatus is reset at that point. Experiment numbers are shown at the right of each record. Expts. 19 and 15 show the absence of light-growth reactions to white light of the type found in coleoptiles.

matter of hours. By resting the switch on top of plants with pronounced plumular hooks, it was shown that the responses of undamaged plants were no different from those of plants punctured by the switch pin. Fig. 9 illustrates parts of some specimen records. The full sensitivity and resolution of the apparatus enables a minute-by-minute record of extension rate to be plotted against time, but nothing at present intelligible appears in the detail available. There is no reaction to light comparable to that of *Avena* (see Went, 1941) nor is there any short term response to the stimulus of resetting the mechanism, other than a general increase in the extension rate, just visible in Expt. 8, and described more fully below.

These preliminary results therefore led to a change in the presentation of the record from a minute-by-minute rate/time graph to one representing the

average rate per successive 10-minute period. This gives a graph similar in appearance to a histogram, and several hours' growth may be shown on one page. It was found that the responses of the plant to light and to mechanical stimulation were of such duration that this method of plotting gave a most

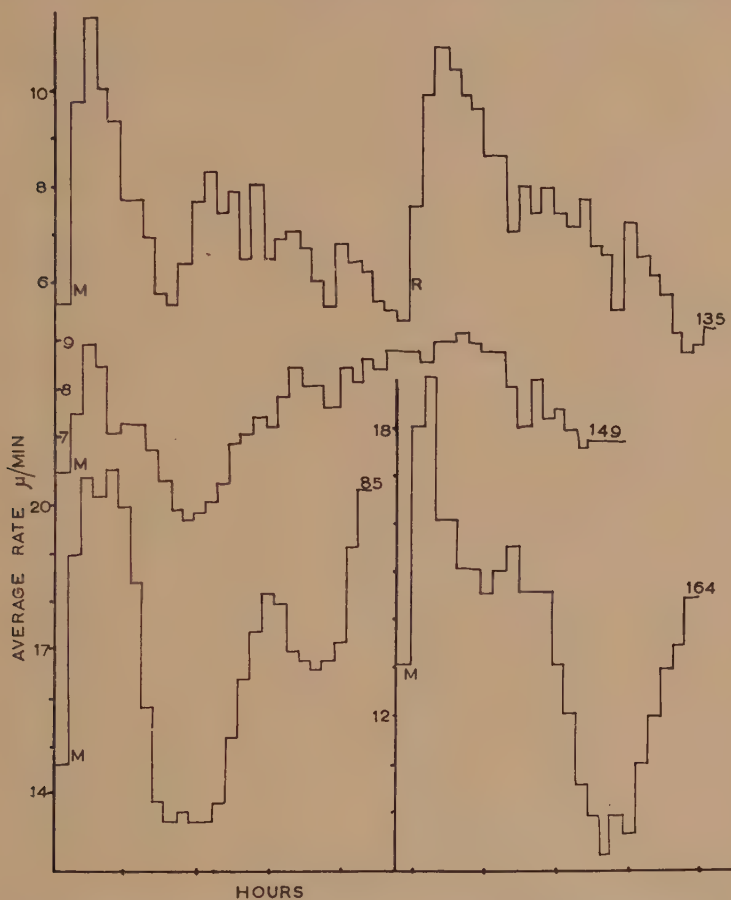


FIG. 10. Long-term observations of the extension rate after mounting of dark grown *Vicia faba* plumules (see text). M, time at which the plant is mounted in the apparatus; R, time at which the apparatus is reset. The experiments are numbered at the right of each graph. Expts. 85, 149: stem clip mounts. Expts. 135, 164: seed clip mounts.

convenient result, combining sufficient resolution on the time scale with a very sensitive indication of variations in the overall extension rate, unconfused by the short-term fluctuations.

(v) *The response to mounting and resetting.* The stimulation afforded by the mounting operation is complex, consisting of slight pulling and bending of both root and shoot. The change in the water-supply from capillary attraction to total immersion probably represents the main factor disturbing the uptake

of water by the root, and the root hairs must suffer damage in the process of removal from the germination jar. Manipulation of the shoot with the fingers and penetration by the switch pin, may both be expected to influence the growth processes of the epicotyl. The net result of all these factors may be

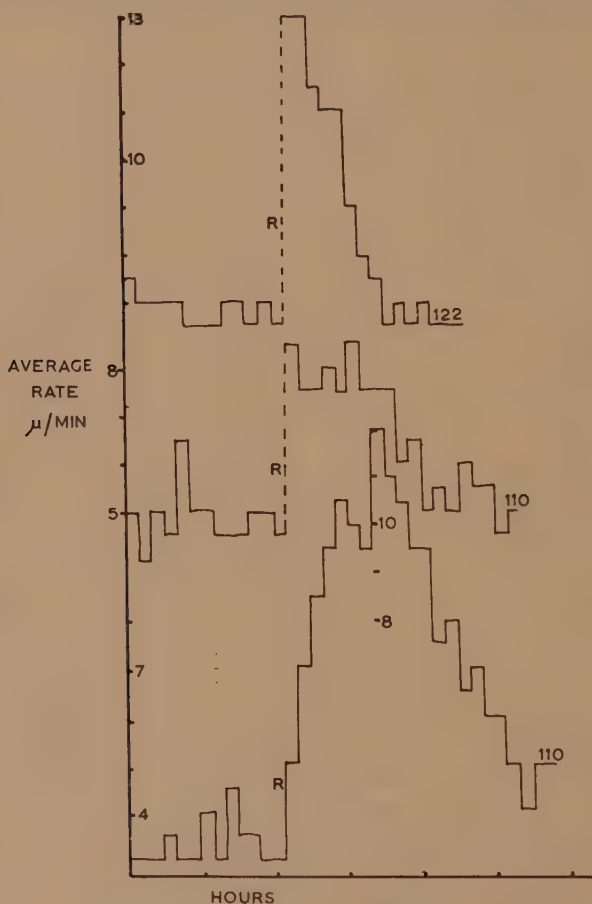


FIG. 11. Response to resetting the apparatus. R indicates the moment of stimulation. Expt. 122. 2nd internode etiolated *Vicia faba* stem. Stem clip mount. Expt. 110. In this, the same plant was observed continuously for some days by repeated re-adjustment of the position of the stem in the clips. The centre graph of the Fig. is taken from an early part of the whole record, and the lower graph shows a greatly increased sensitivity in tissue that developed later.

seen in Fig. 10, which shows a variety of records obtained when using both the seed clip and the stem-clip techniques. Examination of these records shows that amongst a great diversity of response there is a general pattern common, in some if not all features, to each. After mounting, the extension rate is slow for a few minutes, rapidly rising to a peak after some 30 minutes

and thereafter declining to a rate in general less than that finally reached after about 4 or 5 hours. It has been found that as the severity of treatment increases, the form taken by the response becomes more and more clearly of the type described. It is also clear that an experiment involving extension growth

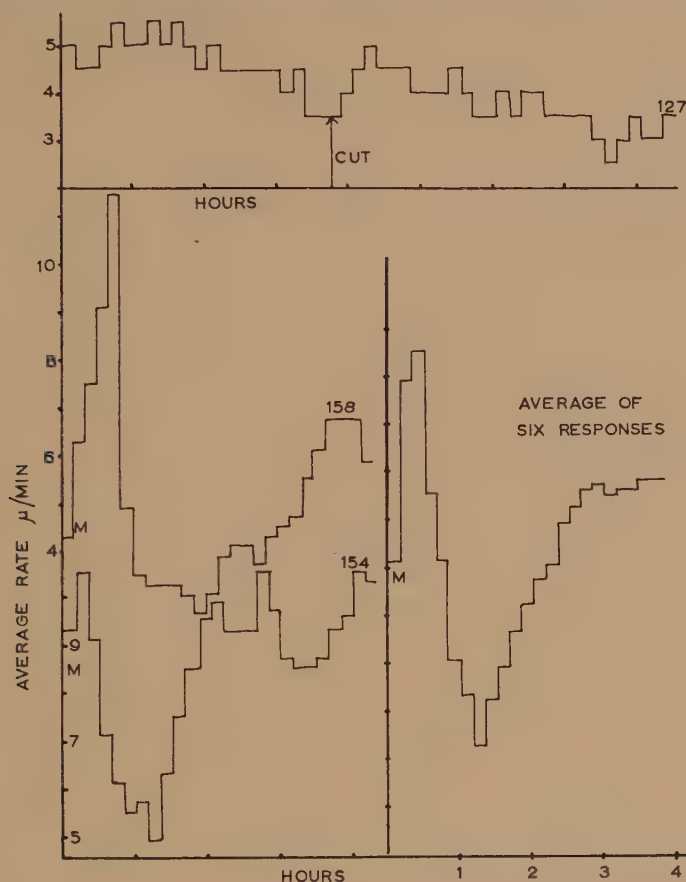


FIG. 12. The response to mounting when the stem is cut from the seed and root. Above: lack of response to cutting the stem under water when already mounted by the stem-clip method. Below: response to mounting when the stem is cut from the seed and root in air and then mounted by the stem-clip method, and supplied at the cut end with water (see text).

must in this material allow at least 5 hours to elapse after mounting, so that these responses may pass. This applies equally to stems isolated from the rest of the plant, and to isolated sections (Idle, 1955).

The response to resetting is also characteristic, and may indicate to some extent the composite nature of the mounting response. This resetting operation consists of raising the cutting head bearing the switch by means of the release nut built in the microtome, whilst keeping the plant in contact with

the switch pin, so that the switch is opened wide. The ratchets of the advancing mechanism are then released, and the toothed wheel rotated in a direction opposite to its normal course, so that the screw mechanism lowers the body of the switch, closing the contacts again. The apparatus will then run for further traverse of the screw. The stimulation given by this operation consists of slight bending of the stem, and movement of the pin in the tissue of the plant. The effect is a rapid rise in the extension rate to a value perhaps double the original, followed by a slow decline lasting up to 3 hours. It may be possible to compare this effect (Fig. 11) with the mounting response, and to attribute the initial short depression and the trough at 2–3 hours of the latter to water-uptake phenomena in the root. Expt. 135 (Fig. 10) shows how similar the two responses may sometimes be. Other preliminary results, such as Expts. 6 and 7 (Fig. 9) suggest that the main factor giving the resetting response is the motion of the pin, for in these experiments when the pin was not used a resetting response was not found, or was very small.

A further indication of the part played by water-uptake phenomena in determining the form of the subsequent response is given by some records obtained from isolated stems. The act of cutting the stem under water does not produce a marked effect on the rate of extension, but if the stem is isolated by a cut in air and mounted by the stem-clip technique, the response is definite. Fig. 12 illustrates the more constant form of this response compared with that of whole plants. Experiments 154 and 158 are typical of some test results, most of which are intermediate with these in amplitude and timing. In these circumstances of similarity of form and of synchronization it is possible to compound several results into a mean by a process of repeated averaging of the extension rates for successive 10-minute periods, starting from the moment of stimulation. An example of a mean of this sort is given in Fig. 12. It represents the changes in the extension rate of dark grown stems when cut from the seed and root with a razor, and otherwise left intact, but the same changes may be seen in a stem section isolated from both root and apex. Simple decapitation gives rise to no definite response other than a gradual reduction of the extension rate to zero in about 15 hours.

ACKNOWLEDGEMENTS

The design and construction of the auxanometer was carried out under a Research Grant of the University of Southampton, and the experimental work continued at Southampton under a grant from the D.S.I.R. The material of this paper is part of a thesis for the degree of Ph.D. of the University of Southampton, and was directed by Professor W. T. Williams, whose help and encouragement I enjoyed throughout.

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Studies in the Respiratory and Carbohydrate Metabolism of Plant Tissues¹

VIII. AN INHIBITION OF RESPIRATION IN PEAS INDUCED BY 'OXYGEN POISONING'

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Received 21 January 1956

SUMMARY

A pressure of oxygen of 5 atmospheres has been shown to inhibit markedly the carbon-dioxide output of green shelled peas. Accompanying this decrease, an accumulation of pyruvic and 'citric' acid has been noted, whilst a depletion in the concentration of α -ketoglutaric acid and, in some experiments, of malic acid occurred. The results have been interpreted as indicating that high pressures of oxygen interfere with the metabolism of citric acid causing a 'jamming' of the tricarboxylic acid cycle; thus, under the conditions prevailing during the oxygen treatment, the tricarboxylic acid cycle is considered to be a major respiratory pathway in peas.

INTRODUCTION

PARTIAL pressures of oxygen greater than one atmosphere produce a variety of injurious effects such as the inhibition of oxygen uptake which have been classified under the general term of 'oxygen poisoning' (Bert, 1878; Stadie *et al.*, 1944, 1945, *a* and *b*; Bean, 1945).

Experiments with homogenates (Elliott and Libet, 1942) and with isolated enzyme systems (Hellerman *et al.*, 1933; Stadie *et al.*, 1945 *c*, *d*, *e*, *f*, and Dickens, 1946, *a* and *b*) have demonstrated that certain enzyme systems involved in respiration are particularly sensitive to oxygen poisoning.

An inhibition of the respiration of plant tissues by partial pressures of oxygen higher than one atmosphere has also been shown (Johannsen, 1888; Caldwell, 1931, 1956). Injury by exposure to oxygen at a pressure of one atmosphere has been noted in potato sprouts (Kidd, 1919), coleoptiles of *Avena* (Albaum *et al.*, 1940, 1942) and pea roots (Galston and Seigel, 1954).

Further Barker and Mapson (1955) have shown that prolonged exposure of potato tubers to one atmosphere pressure of oxygen reduced the carbon-dioxide output with increases in pyruvic and 'citric' acids and decrease of α -ketoglutaric and malic acids which were ascribed to a 'block' in the tricarboxylic acid cycle at the citric acid stage.

Moreover, the growth and oxygen uptake of the aerial mycelium of the actinomycete *Micromonospora vulgaris* are inhibited by oxygen at a pressure

¹ For earlier numbers of this series see *Proc. Roy. Soc. B.* 140 to 143.

of one atmosphere and keto compounds (possibly pyruvic acid) accumulated during the inhibition (Webley, 1954).

It seems likely therefore, that the depressant effect of oxygen pressures on respiration for both animal and plant tissues is due to the inhibition of certain enzymic systems and the primary objectives of our work with peas has been to ascertain whether the tricarboxylic acid cycle is functional in this material.

METHODS

The peas were gathered from field sowings at the time of the normal commercial harvest.¹ After shelling and sampling, some samples were placed in sealed jars through which flowed currents of carbon-dioxide-free air at from 4 to 8 litres per hour, depending on the weight of the sample. The remaining samples, to be subjected to oxygen at high pressure, were held in pressure cookers through which passed either carbon-dioxide-free air or oxygen at the rate just mentioned. The technique of Caldwell (1931; 1956) was used with only slight modification. The experiments were conducted in a constant-temperature room at 15° C.

In all the experiments described in this paper, the high-pressure treatment was with oxygen at a pressure of 5 atmospheres. Before use, pressure cookers which had been adapted to serve as pressure vessels were tested at a pressure of 6 atmospheres and for safety were kept in wooden boxes.

pH measurements

The pH was determined using a glass electrode on water extracts made by grinding the peas in distilled water with sand.

Hydroxy and keto-acids

The methods used for the determination of the malic and 'citric' acid fractions were based on the titrimetric procedure of Isherwood and Hanes (1953) and were similar to those used by Barker and Mapson (1955) except that fresh whole peas were taken for analysis and blended in the 10 per cent. w/v trichloroacetic acid. The term 'citric' acid is used as this fraction would contain *cis*aconitic and *isocitric* acids if these were present.

Keto-acids

The method used in killing the tissue and in extracting the keto-acids was based on that devised by Isherwood and Niavis (1954).² The chromatographic estimation of the 2:4-dinitrophenylhydrazones followed the method of Isherwood and Cruickshank (1954).

Weighed samples of whole peas (about 10–15 g.) were dropped into 30 ml. of boiling methanol for 3 minutes. The methanol was poured off, concentrated under reduced pressure at 30° C. and combined with the main extract (see

¹ We wish to thank Mr. H. G. Randall of Wood Hall Farm, Balsham, and Mr. R. C. Bradnam of Milton who kindly allowed us to collect peas from their farms.

² For later developments in the killing and extraction procedure see Isherwood and Niavis (1956).

later). Meanwhile the peas were homogenized for 3 minutes with 50 ml. 0.2 M. NaH_2PO_4 solution. The homogenate was carefully heated to 40° C. allowed to stand for 5 minutes and then centrifuged. The supernatant was poured off, and the residue twice resuspended in 40 ml. 0.2 M. NaH_2PO_4 heated as before and again centrifuged. The protein was then removed by adding 8 per cent. metaphosphoric acid to the combined supernatants together with the concentrate (final concentration of metaphosphoric 5 per cent. v/v).

The protein-free extract was incubated at 30° C. for 25 minutes with 2:4-dinitrophenylhydrazine (0.5 per cent. in 5 N. H_2SO_4). The phenylhydrazones were successively extracted into 20 per cent. v/v ethanol/chloroform and 10 per cent. sodium carbonate. To reduce emulsification, 30 per cent. v/v of 95 per cent. ethanol may be added to the extracts. The carbonate extract was acidified (pH 2) with 2 N. H_2SO_4 . Finally the phenylhydrazones were extracted into 20 per cent. v/v ethanol/chloroform and the extracts taken to dryness under reduced pressure at 30° C. The residue was dissolved in the minimal amount of glacial acetic acid for chromatography.

For the estimation of pyruvic acid Whatman No. 2 paper buffered with phosphate buffer (0.2 M. KH_2PO_4 , 0.2 M. Na_2HPO_4 , pH 6.2) was used and the chromatograms developed with tertiary amyl alcohol/ethanol/water (50/10/40 v/v). For α -ketoglutaric acid, Whatman No. 3 paper was buffered with 0.1 N. glycine/NaOH, pH 8.2, and the chromatograms were developed with tertiary amyl alcohol/propanol/0.88 ammonia 65/5/30 v/v.

EXPERIMENTAL RESULTS

Expt. 1. Laxton's Superb peas picked on 5 August 1954 (Figs. 1 and 2). For this experiment, 12 samples, each of about 1,000 g., of Laxton's Superb peas, were provided. Six of these samples were used as air controls; the remainder, after a preliminary period of one day in air, were exposed to oxygen at a pressure of 5 atmospheres. Typical curves of the carbon dioxide output (measured over 6-hour periods) of one air control and one 'oxygen sample' are given in Fig. 1.

The preliminary decrease in the observed rate of carbon-dioxide output of the 'oxygen sample' is due primarily to the change in the physical conditions of the system (Caldwell, 1931, 1956) and was followed by a temporary increase almost to the air line (Fig. 1). The pH decreased slowly in the air controls, and quickly in the 'oxygen samples'. The changes in pyruvic, 'citric', α -ketoglutaric and malic acids in the air control and oxygen samples are shown in Fig. 2.

The content of malic and α -ketoglutaric acids decreased initially in the oxygen samples; the other acids increased in the oxygen samples while remaining roughly constant in the controls.

In all the experiments reported in this paper, injury was caused to the samples in the later stages of the oxygen treatment. The peas developed a spongy texture and there was a progressive bleaching of the chlorophyll. In

the final stages, exudation occurred and a 'stale' smell developed. Injury caused by treatment with high pressures of oxygen has also been reported in apples by Caldwell (*loc. cit.*).

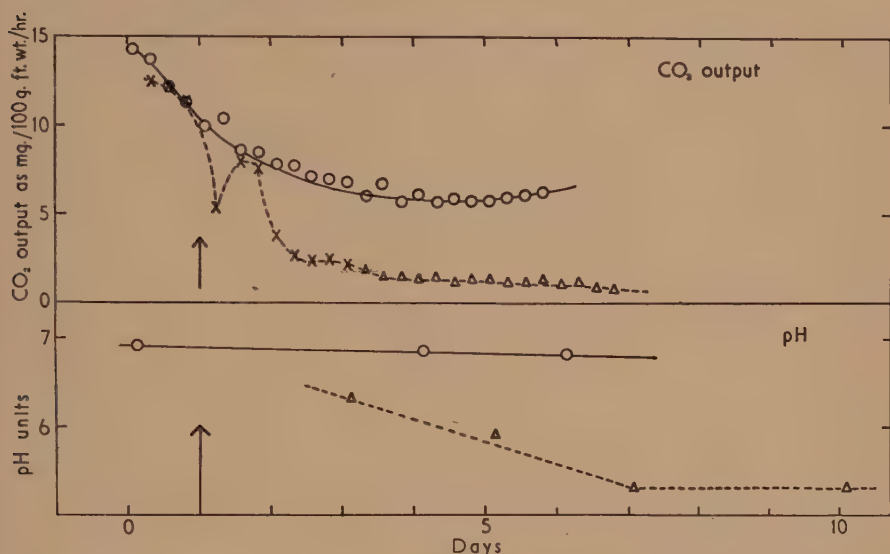


FIG. 1. *Expt. 1.* Laxton's Superb peas. Changes at 15° C. in the rate of carbon-dioxide output and pH for samples held in air (continuous line) and for samples subjected to oxygen at 5 atmospheres pressure (broken line).

Expt. 2. Onward peas picked on 16 August 1954 (Figs. 3 and 4). In this experiment peas of another variety, Onward, were used. A sample was taken for analysis after only 24 hours from the application of high pressure in order to determine the initial effects of the treatment.

At the time of the first analysis of the oxygen-treated samples both pyruvic and 'citric' acids had increased in concentration while α -ketoglutaric and possibly malic acid had decreased as compared with the content of the air controls (Fig. 4). The subsequent changes in pyruvic and α -ketoglutaric acids were similar to those in *Expt. 1* (Fig. 2). In contrast, however, with the results of that experiment, after 6 days of oxygen treatment the content of 'citric' acid decreased while malic increased above the value for the air controls. Another contrast is that there is no evidence of a lag in the increase of 'citric' acid in the oxygen samples as occurred in *Expt. 1*.

Expt. 3. Onward peas picked on 6 September 1954 (Figs. 5 and 6). In this experiment, also with Onward peas, samples were taken daily for analysis in order to define more closely the changes during the early stages of the oxygen treatment.

The curves for the carbon-dioxide output of an air-control sample and for two oxygen-treated samples are given in Fig. 5, and are similar to those obtained in the previous experiment. Acid concentration data are given in Fig. 6.

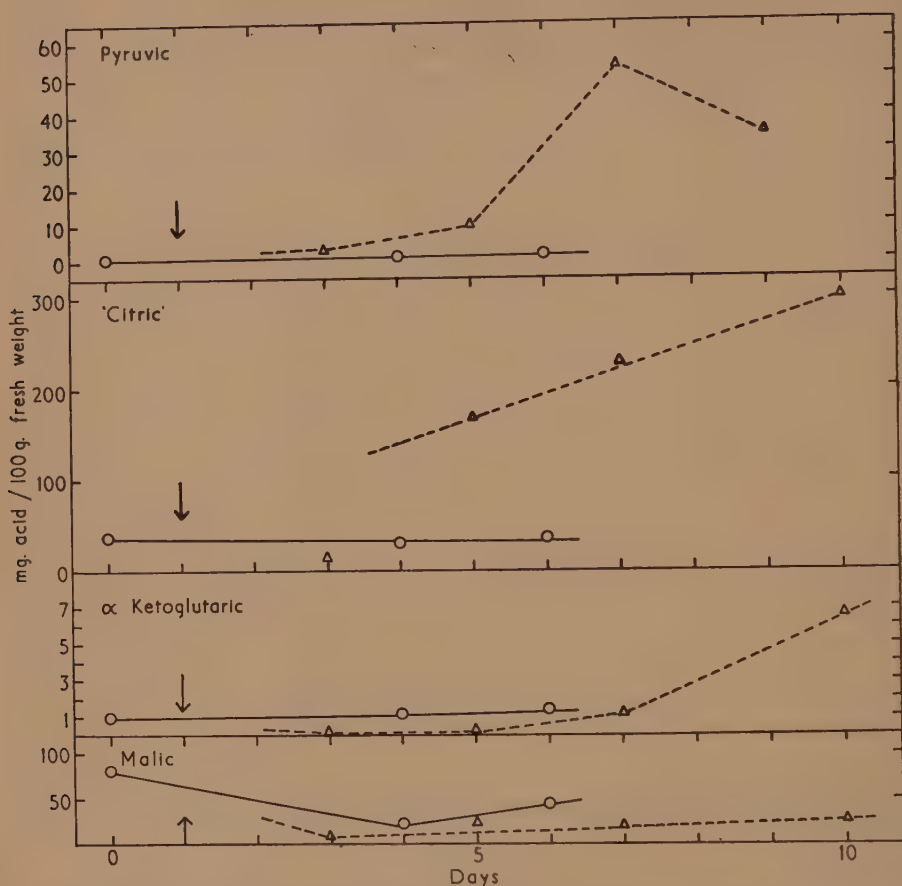


FIG. 2. *Expt. 1.* Laxton's Superb peas. Changes at 15° C. in air (continuous line) and in oxygen at 5 atmospheres pressure (broken line) in the contents of pyruvic, 'citric', α -ketoglutaric, and malic acids.

DISCUSSION

The evidence presented shows that the application of oxygen at a pressure of 5 atmospheres leads to a rapid inhibition of the carbon dioxide output from green peas. During the initial stages of the inhibition, both pyruvic and 'citric' acids increased whilst α -ketoglutaric and, to a lesser extent, malic acid, decreased in concentration.

The initial rapid rise in 'citric' acid suggests that high pressures of oxygen interfere with the subsequent metabolism of this acid. The behaviour of the other acids is in harmony with what would be predicted if it is assumed that the main pathway for the oxidation of pyruvic and 'citric' acids is via the tricarboxylic acid cycle. Interference with the oxidation of 'citric' acid would be expected to increase its concentration, perhaps with associated increases in the pyruvic acid content due to the jamming of the cycle. Similarly, acids

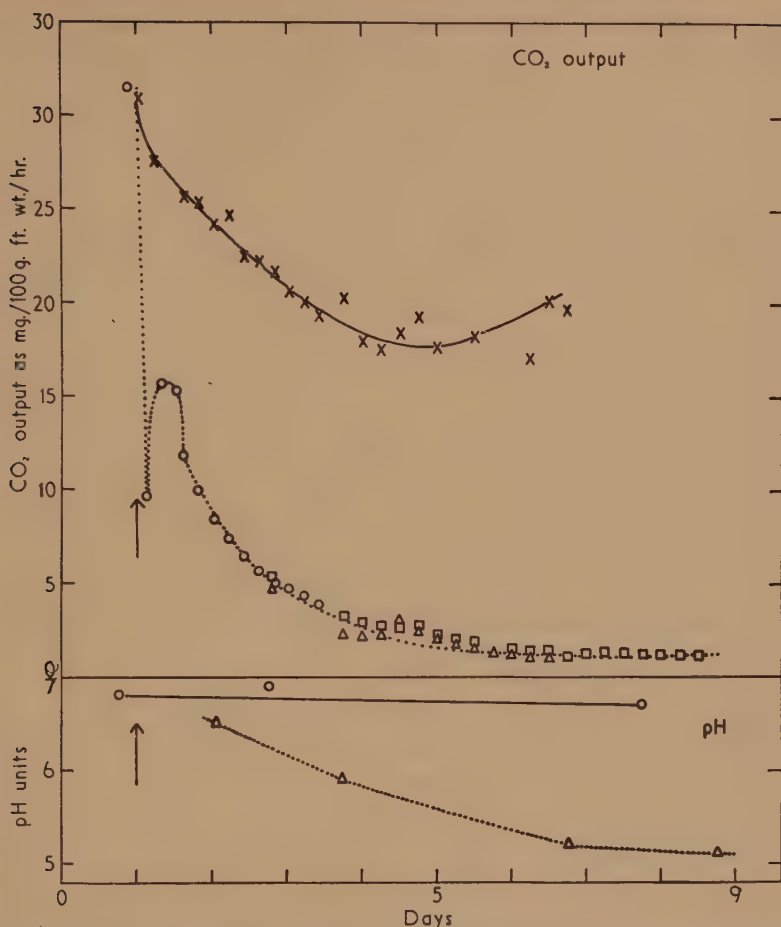


FIG. 3. *Expt. 2.* Onward peas. Changes at 15° C. in the rate of carbon dioxide output and in pH for samples held in air (continuous line) and for samples subjected to oxygen at 5 atmospheres pressure (broken line).

occurring after citric in the cycle would be expected to decrease in concentration. For α -ketoglutaric and for malic acids, this depletion was observed and the results may therefore be cited as evidence for the occurrence and function of the cycle in green shelled peas under the conditions prevailing in the experiments.

With prolonged oxygen treatment it is to be expected that progressively more aspects of metabolism would be affected, resulting ultimately in the disorganization and death of the tissue, as recorded above. The decrease in pyruvic and 'citric' acids and the increase of α -ketoglutaric and malic acids noted in the later stages of some of the experiments may thus be ascribed to secondary changes following the primary 'block' in the tricarboxylic acid cycle which occurs in the initial stages of 'oxygen poisoning'.

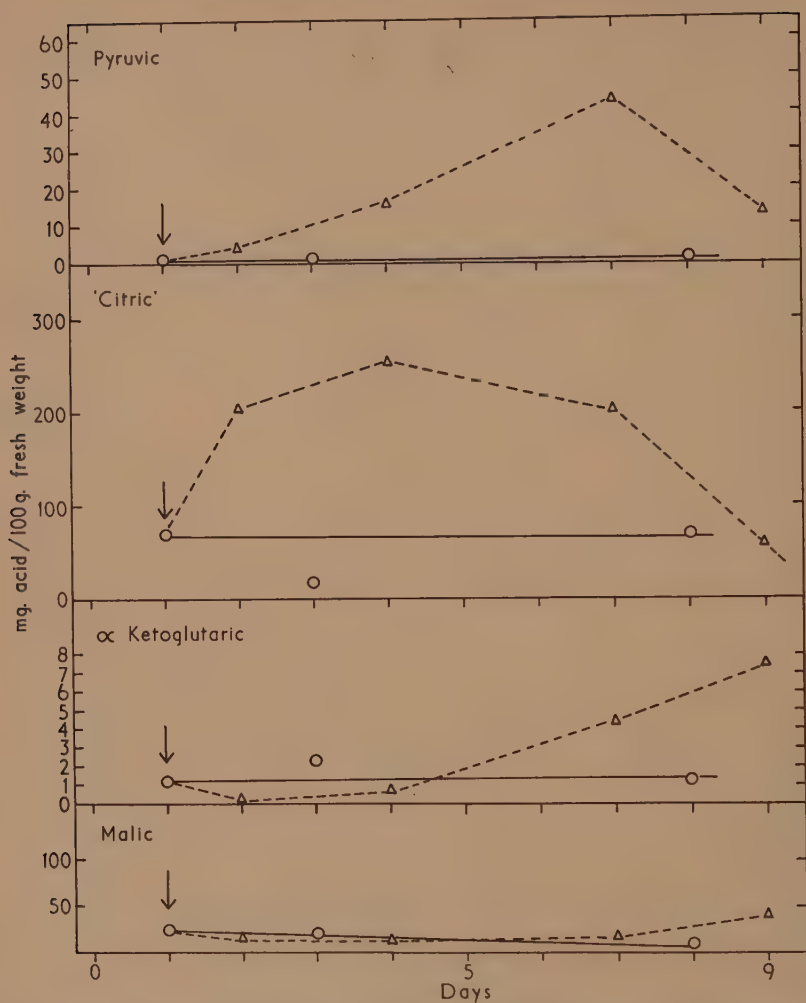


FIG. 4. *Expt. 2.* Onward peas. Changes at 15° C. in air (continuous line) and in oxygen at 5 atmospheres pressure (broken line) in the contents of pyruvic, 'citric', α -ketoglutaric and malic acids.

As mentioned, the method used for the determination of 'citric' acid does not differentiate between citric and *isocitric* acids. Consequently the evidence presented above merely indicates that high pressures of oxygen cause a block in the cycle between citric and α -ketoglutaric and gives no indication whether aconitase or *isocitric* dehydrogenase is the enzyme involved in the inhibition.

It is not intended here to discuss the mechanism of inhibition which may be connected with enzyme sulphhydryl groups. Citric acid could accumulate if either aconitase or *isocitric* dehydrogenase were inhibited by oxygen.

Either or both enzymes might be expected to be susceptible to 'oxygen poisoning'. For maximum activity *in vitro*, preparations of aconitase have been

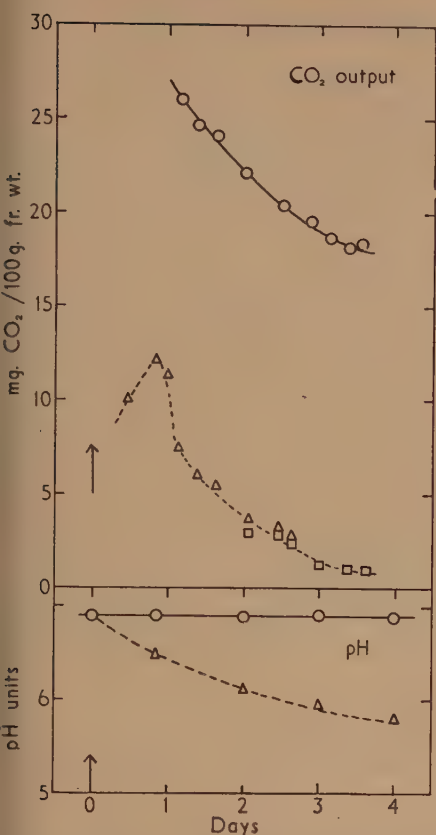


FIG. 5. *Expt. 3. Onward peas.* Changes at 5° C. in the carbon dioxide output and in pH for samples held in air (continuous line) and for samples subjected to oxygen at 5 atmospheres pressure (broken line).

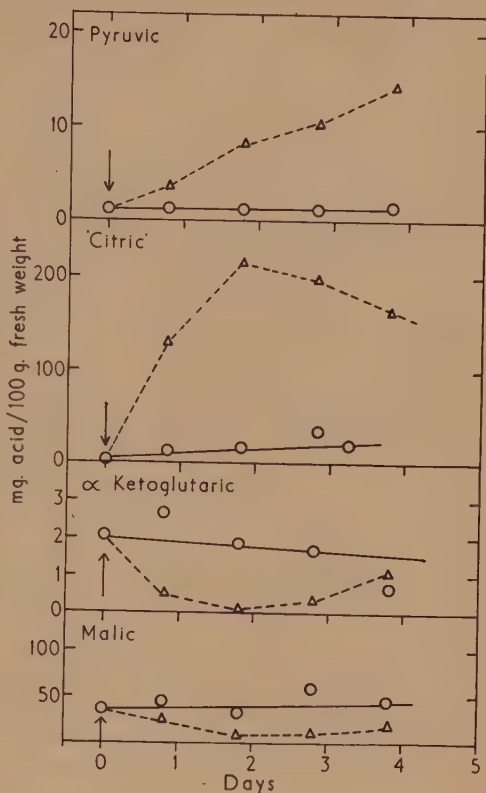


FIG. 6. *Expt. 3. Onward peas.* Changes at 15° C. in air (continuous line) and in oxygen at 5 atmospheres pressure in the contents of pyruvic, 'citric', α-ketoglutaric, and malic acids (broken line).

shown to require the presence of ferrous ions and cysteine (Dickman and Cloutier, 1950, 1951; Morrison, 1954, *a* and *b*) and isocitric dehydrogenase has been reported to possess a free —SH group although of somewhat unusual properties (Lotspeich and Peters, 1951). Recently Davies (1955) has extracted isocitric dehydrogenase from pea mitochondria and has shown that the activity of the preparation decreases rapidly in air. Reactivation may be brought about by addition of reducing agents.

In the experiments reported above, the rate of carbon dioxide output falls rapidly in the air-control samples with, in general, little change in the concentration of the keto or hydroxy acids. The marked alterations observed in our experiments in the contents of the various acids during treatment with oxygen demonstrate conclusively that under these conditions the tricarboxylic acid cycle is a major pathway in respiration. Further evidence is, however, required to establish whether or not the cycle is also of major importance in

peas when not subject to the conditions prevailing in the oxygen-treated samples.

ACKNOWLEDGEMENTS

This work has been carried out under the general direction of Dr. J. Barker, F.R.S. and we wish to express our thanks to him for his advice and encouragement and also for help with the drafts. We are also indebted to Dr. F. A. Isherwood, Mr. C. A. Nias, and Dr. H. G. Wager for advice about the experimental methods.

The research was financed by a grant for a special research awarded by the Department of Scientific and Industrial Research. This assistance is gratefully acknowledged, as is also the interest and encouragement shown by the Agricultural Research Council.

We wish to thank Miss R. A. Barrett, Miss J. A. Nightingale, and Mr. B. L. Chapman who helped with the analyses.

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The Suitability of Some Algae for Mass Cultivation for Food, with Special Reference to *Dunaliella bioculata*

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Received 12 February 1956

Twenty-five strains of freshwater and saltwater algae have been investigated for their suitability for mass cultivation for food. Under laboratory conditions a strain of *Dunaliella bioculata* has been found to give yields comparable with those obtained from *Chlorella*. It is suggested that only when the growing algal cells are exposed in very thin layers will the yield not be limited by light penetration. The storage polysaccharide of *D. bioculata* contains a 1:4-glucosan resembling starch. All the essential amino-acids have been shown to be present with the possible exception of methionine or valine and tryptophane.

INTRODUCTION

THE possibility of supplementing food-supplies by the mass cultivation of algae has received considerable attention during the past few years (Burlew 1953). Little consideration, however, has been devoted to the choice of pre-eminently suitable organisms for the purpose. Much experimental work has been done, both on laboratory and pilot-plant scales, for the most part with species of *Chlorella*, especially *Chl. pyrenoidosa* which possesses a high growth-rate and can attain high population densities: populations with a dry weight of 55 g./l. with a daily yield of 2.45 g./l. having been reported (Myers, Phillips, and Graham, 1951). There is, however, no good reason to believe that it is unique in these respects, because no comparable information for other algae is available and until recently few data existed comparing *Chlorella* with other algae under ordinary laboratory conditions. Even now the field has barely been touched (Ketchum, Lillick, and Redfield, 1949; Pruess *et al.* 1954) and it is still too early to conclude that a species of *Chlorella* will be the organism whose advantages most outweigh its disadvantages. A survey of still more genera, and especially marine forms, might well produce types showing promising growth-rates and population densities but without one or more of the disadvantages of *Chlorella*. Work along these lines is described in this paper.

Selection of strains for investigation. The largest collections of algae in the United Kingdom are those maintained at the Plymouth Marine Laboratory (sea-water forms) and at the Botany School, Cambridge (mainly freshwater forms). The types used in this work were obtained from the latter source, but

courtesy of Mr. E. A. George, though in several cases the marine strains had come originally from Plymouth.

The freshwater forms which were considered included *Ankistrodesmus convolutus* (Cambridge catalogue No. RF 2), *A. falcatus* var. *spirilliformis* (4b), *Chlamydomonas eugametos* (11/5 a and b, 11/6 a and b), *C. moewusii* (11/16 f), *C. pulsatilla* (11/44), *Dictyosphaerium pulchellum* (222/2), *Euglena gracilis*, *Pandorina morum* (60/1), and *Volvox tertius* (88/3). The strains of *Ankistrodesmus* and *Dictyosphaerium*, the latter a mucilaginous form, showed promising growth and were therefore selected for further study.

Of marine algae, attention was first drawn towards the genus *Dunaliella* (*Volvocales*), whose members are usually described as being naked, by a report that one species (*D. media*) attained a population of 9.6×10^6 cells/ml. and had a high growth-rate (Lerche, 1936-7). Five strains were available, one each of *D. eugametos* (19-1), *D. piercei* (19-2), *D. salina* (19-3), *D. bioculata* (19-4), and *D. primolecta* (11/34).¹ In preliminary experiments, *D. bioculata* and *D. primolecta* could be seen to grow much better than the other strains and they were therefore chosen for further investigation. Visual comparisons of the growth in test-tubes of other marine types were made but none showed a promisingly high rate of multiplication. They included *Chlamydomas* sp. (11/35), *Euglena viridis* var. *maritima* (1224/17 d), *Nitzschia closterium* f. *minutissima* (1052/1 a)—possibly more correctly *Phaeodactylum tricornutum*, *Platymonas subcordiformis* (161/1 a and b), *Platymonas* sp. (66/1 a and b), and *Porphyridium cruentum* (1380/1 a).

METHODS

It is clearly impracticable to begin comparing the growth-rates of algae by determining experimentally for each the most suitable culture medium. Instead, it is necessary to begin by using the media on which they are maintained in the culture collection and which have been shown to support at least moderate growth. However, provided that there is present an adequate supply of nitrogen and phosphorus, the other constituents of the medium can vary within fairly wide limits.

The following media were used, the quantities given being for 1 l.

(a) for *Dunaliella bioculata*—NaCl 60 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 10 g., $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 8 g., KCl 2 g., NaNO_3 1 g., CaSO_4 0.5 g., K_2HPO_4 0.02 g., soil extract 100 ml.

(b) for *D. primolecta*—NaCl 30 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5 g., $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 4 g., KCl 1 g., NaNO_3 1 g., CaSO_4 0.25 g., K_2HPO_4 0.01 g., soil extract 100 ml.

(c) for the freshwater types— KNO_3 1 g., K_2HPO_4 0.01 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g., soil extract 100 ml.

The soil extract was prepared by autoclaving together equal weights of soil and water, allowing the solid matter to settle, siphoning off the super-

¹ This strain was supplied as *Chlamydomonas* I (11/34). It is considered to be a species of *Dunaliella* and is shortly to be described by Dr. R. W. Butcher as *D. primolecta*.

nantant liquid, and filtering through a thin layer of kieselguhr supported on a filter paper in a Buchner funnel.

All the constituents of the media except phosphate were dissolved in less than total volume, filtered, made up to total volume, distributed, and autoclaved at 15 lb. pressure for 15 min. No adjustment of pH was made. When cool the appropriate quantity of sterile 2 per cent. K_2HPO_4 was added.

For most of the experiments the algae were grown in Roux bottles fitted with gas inlet and outlet tubes and each containing 750 ml. of medium. The illumination was provided by four long 40-watt fluorescent lamps (Atlas natural-daylight type) fixed one above the other, their centres 2 in. apart. The bottles were supported vertically in two opposed rows of four, on carriers with polished steel reflectors, mounted so that the middles of the bottles were $5\frac{1}{2}$ in. from the centre of the lamps. The light-intensity incident on the bottles was 150 foot candles. They were shaken gently from side to side to give some mechanical agitation. A stream of air containing 5 per cent. CO_2 was passed through each bottle at a rate of 7 vol./hour to provide CO_2 for photosynthesis and to give further agitation.

The experiments were carried out in a constant-temperature room but it was found that heat from the lamps raised the temperature of the cultures $7^\circ C$. above ambient.

A standard inoculation giving an initial population of 1×10^4 cells/ml. was aimed at in most of the experiments. Samples were removed immediately and at intervals, and total counts were made in a counting chamber under the microscope. To facilitate counting during the early stages of growth the samples were concentrated by centrifuging.

Dry weights were determined by concentrating a suspension of known density and drying aliquots to constant weight first in a hot air oven at $67^\circ C$. and then *in vacuo* over P_2O_5 . After drying, the *Dunaliella* samples were incinerated so that the dry weight could be determined on an ash-free basis.

RESULTS

A. *Determination of growth characteristics.* Growth curves for the algae were obtained at a number of different temperatures: although the actual curves are not given, the relevant data are shown in the Table. The curves were of the usual type consisting essentially of a logarithmic phase of growth leading to a plateau. Generation times were calculated from the logarithmic phase and maximum populations were estimated from the shapes of the curves. The maximum daily yields during the experiment have also been calculated and are expressed in terms of cells/ml. and g. dry weight/l.

Several features of interest emerged. For example, the growth curves showed that, as the temperature was raised, the maximum population decreased and was less at the temperature of most rapid growth than at lower temperatures. The two marine strains of *Dunaliella* had higher maximum growth-rates than the freshwater forms, dividing roughly once every 5.5 as compared with 8.5 hours. They also had higher optimum temperatures than

TABLE
Generation times, maximum populations, and daily yields of several algae over a range of temperatures

Temp. °C.	Generation time (hr.)			Maximum population (cells/ml.)			Maximum daily yield							
	Ankistrodesmus RF2	Ankistrodesmus 4b	Dictyosphaerium 222/2	<i>Dunaliella bioculata</i>	<i>D. primolecia</i>	Ankistrodesmus RF2	Ankistrodesmus 4b	Dictyosphaerium 222/2	<i>Dunaliella bioculata</i>	<i>D. primolecia</i>	Cells/ ml.	g. dry wt./l.	Cells/ ml.	g. dry wt./l.
17	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25	8.3	13.5	8.3	14.5	11.3	—	—	—	—	—	—	—	—	—
29	10.3	8.4	9.0	9.0	8.3	1 × 10 ⁸	7 × 10 ⁷	5 × 10 ⁷	2.5 × 10 ⁷	1.2 × 10 ⁷	3.5 × 10 ⁶	0.23	1.4 × 10 ⁶	0.18
33.5	65	—	9.0	5.7	5.4	—	—	2.5 × 10 ⁷	1.2 × 10 ⁷	7 × 10 ⁶	2.4 × 10 ⁶	0.16	2.0 × 10 ⁶	0.24
36.5	—	—	—	6.3	9.5	—	—	—	7.2 × 10 ⁶	4 × 10 ⁶	3.0 × 10 ⁶	0.20	1.6 × 10 ⁶	0.19
	—	—	—	—	—	—	—	—	—	—	2.5 × 10 ⁶	0.17	9.5 × 10 ⁵	0.11

the freshwater forms, the latter dividing most rapidly in the range 25–29° C. and the former at 33.5° C.

Yield, as measured in terms of the daily increase in population density or dry weight, is a function of both rate of growth and number of cells. Although they have a high growth-rate, the marine strains give low yields because of their low ultimate population densities. In the following section, experiments are described in which attempts were made to find and, if possible, remove the factors limiting the maximum yield obtainable from *Dunaliella bioculata*.

B. *Investigation into the factors limiting the daily yield obtainable from Dunaliella bioculata.* The first factors investigated were the sufficiency of the culture medium used and the presence or absence of inhibitory substances produced by the alga itself which might be limiting the population. Algae were grown in Roux bottles until growth had virtually ceased. The cells were removed by filtration and discarded: the medium was resterilized and re-inoculated. When the second growth had reached its limit, supplements of soil extract, KNO_3 , K_2HPO_4 , and $\text{KNO}_3 + \text{K}_2\text{HPO}_4$ were added to different bottles. The results are shown in Fig. 1. Clearly phosphate was limiting the density of the second growth. Accordingly, fresh Roux bottles were inoculated and further supplies of nitrate and phosphate added during growth. However, the same population was attained as in control flasks, indicating that phosphate supply cannot be the only factor limiting the population density.

The fact that, after filtering off the first crop and reinoculating, the addition of more phosphate resulted in a second crop of similar magnitude to the first is taken as evidence that growth was not limited by some substance produced by the alga itself.

The possibility that the yield was being limited by an inadequate CO_2 supply was next tested. The algae were grown in 1½-in. diameter test-tubes in a narrow, glass-sided water-bath illuminated from both sides by pairs of 40-watt fluorescent lamps. The phosphate concentration in the medium was increased 10-fold and the nitrate concentration doubled. Under these conditions a growth-rate was obtained similar to that in Roux bottles. When the population had reached about 10^6 cells/ml., one-third of the tubes had the gas flow increased from 7 to 14 vols./hour and one-third had the normal gas mixture of 5 per cent. CO_2 in air replaced by one of 10 per cent. CO_2 in air. The remainder were left unchanged as controls. The same growth curves were obtained in all tubes, indicating that CO_2 supply was not limiting.

Similar experiments were carried out to test the effect of increased light intensity, conditions being so arranged, on the basis of the previous experiments, that growth over the range in question was not limited by nutrients or by CO_2 supply. When growth had reached 10^6 cells/ml., half the tubes had their light intensity increased from 100 to 400 foot candles by the addition of two 80-watt spherical fluorescent lamps (Ediswan Escura type). The results of experiments with increased light intensity carried out at 25°, 30°, and 35° C. are shown in Fig. 2: the daily increases in population are given in addition to the growth curves.

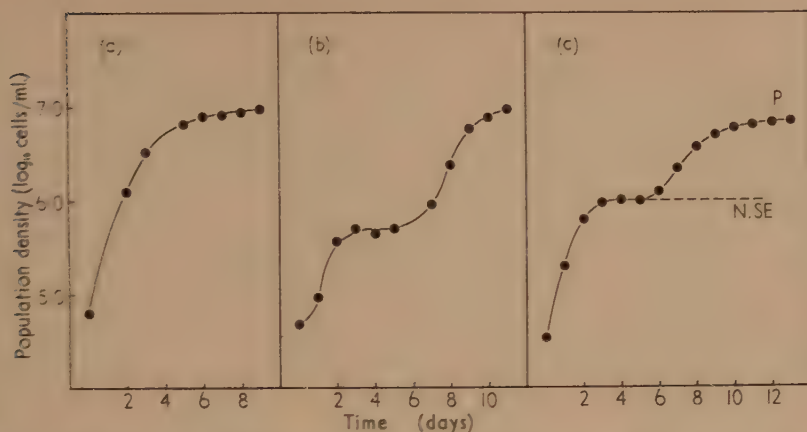


FIG. 1. The effect of the addition of nitrate (N), phosphate (P), and soil extract (SE) to a reinoculated batch of medium after the removal of one crop of *Dunaliella bioculata* cells. (a) First crop. (b) Addition of N, P, and SE together after cessation of growth in reinoculated medium. (c) as (b) but N, P, and SE added separately.

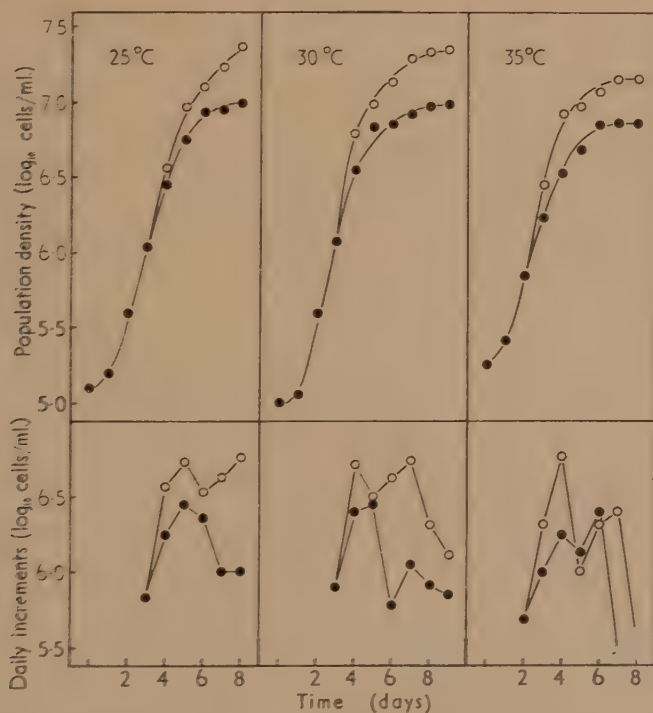


FIG. 2. The effect of temperature and light intensity on the growth curve and daily yield of *Dunaliella bioculata*. (●—●) 100 foot candles; (○—○) 400 foot candles.

The 4-fold increase in light intensity resulted in no appreciable increase in growth-rate but the logarithmic phase of growth was prolonged so that when the experiment was terminated the tubes which had received the higher light intensity had roughly twice the population density of the controls. It is assumed, because no other factor was known to be limiting, that the cessation of logarithmic growth in these experiments occurred when the rising population density prevented all the cells from being adequately illuminated. This is suggested by the rough proportionality between the superficial light intensity and the cell density at which growth ceased to be logarithmic; at 100 f.c. light became limiting at a density of about 1.6×10^6 cells/ml. while at 400 f.c. it became limiting at about 8.5×10^6 cells/ml. This is reflected in the increased yields. At the same time, however, the efficiency of conversion fell. The illuminated surface of each tube was 120 cm.², so that at 100 f.c. each tube received about 2.5 k.cal./day (approximately 0.5 g. dry wt./day) and at 400 f.c., 10 k.cal./day (approximately 2.0 g. dry wt./day). The yields actually obtained were of the order of 0.014 g. dry wt./day at 100 f.c. (2.7 per cent. efficiency) and 0.027 g. dry wt./day at 400 f.c. (1.4 per cent. efficiency).

In the case of the effect of temperature, the results confirm the earlier observations. The generation times in hours were 13.5 at 25°, 9.8 at 30°, and 12.2 at 35°. This last high value may possibly be due to the fact that the temperature control of the bath broke down at one stage and the temperature rose to at least 38°. Above the optimum temperature the generation time increases rapidly with increased temperature. The maximum population decreased with increased temperature.

Pratt (1938) and Myers, Phillips, and Graham (1951) have shown respectively that β -indolylacetic acid (IAA) and ethylenediaminetetracetic acid (EDTA) increase both the rate of multiplication and the population density of *Chlorella* and it was decided to test their effects on *Dunaliella*. Pratt (1938) found the optimum concentration of IAA to be 50 p.p.m., 100 p.p.m., and 10 p.p.m. being less effective. With *Dunaliella*, no growth occurred in the presence of 50 and 5 p.p.m. IAA, and with 0.5 p.p.m. growth was slightly below that of the control. Nor was any benefit gained with EDTA. The addition of 1 ml. EDTA solution/l. as described by Jacobson (1951) caused some inhibition of growth while 0.1 ml. was without effect.

C. *Chemical composition of Dunaliella bioculata*. A knowledge of the composition of algal material is plainly essential in any assessment of food value. Accordingly a preliminary study has been made of the major constituents of *Dunaliella*, using material accumulated during experiments on growth characteristics not reported here, and harvested when growth was limited by light intensity. The cells were harvested from large glass tanks by centrifuging, and were then dried and stored *in vacuo* over P₂O₅. A gross analysis, carried out by the courtesy of the Director of the National Institute of Agricultural Botany, gave the following percentage contents: Crude protein (N \times 6.25) 48.5; Ether extract 7.6; Ash 4.4; Carbohydrates (by difference) 39.5.

The storage polysaccharide was investigated as follows. The dried material

was disintegrated in a ball-mill for 24 hours and then examined qualitatively, following in outline the method described by Jermyn and Isherwood (in the press) for the quantitative analysis of the cell-wall of the pear. On hydrolysis, all the fractions gave glucose on chromatographic analysis. The only other sugar detected, and then only in traces, was rhamnose. The water-soluble fractions gave a blue-black colour with iodine and were broken down by diastase and salivary α -amylase. The final reducing value of these water-soluble fractions suggested that about 50 per cent. was a 1:4-glucosan such as starch. The other material may have been non-polysaccharide in nature since acid hydrolysis gave a very similar figure for the polysaccharide present.

The insoluble residue from the alga consisted of a resistant polysaccharide giving only glucose on hydrolysis, and which would appear to be cellulose, although the genus is usually described as being naked. There is reason to suppose that about half of the carbohydrate present is of a type which should be broken down by digestive amylases.

The following amino-acids were detected in the protein hydrolysate (5 N HCl for 36 hours): alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, lysine, methionine (and/or valine), phenylalanine, proline, serine, threonine, and tyrosine.

DISCUSSION

The emphasis of this work has been on the testing of a limited number of algae for their suitability as alternatives to *Chlorella* for mass cultivation, rather than on research into the physiology of algal growth and multiplication; *Chlorella* has the advantage that both the rate of multiplication and population density attainable are high, but the daily yield of 2.45 g. dry wt./l. obtained by Myers *et al.* (1951) was obtained under very special conditions, and normal values are of the order of 10 times less. *Dunaliella bioculata* has here been shown to achieve a rate of multiplication somewhat higher than *Chlorella pyrenoidosa* with small maximum population. However, daily yields of 0.37 g. dry wt./l. (ash-free) have been obtained which compare favourably with those obtained from *Chlorella* under similar conditions, i.e. 0.19 g./l./day (Myers *et al.*, 1951). Strains of twelve other species were considered but were found to be less promising.

Two considerations seem to merit amplification; namely the effect of temperature and the effect of light intensity. It is clear from the Table that temperature affects both the rate of growth and the maximum population density. The relationship between growth-rate and temperature is such that as the temperature is increased, the generation time decreases to a minimum (the optimum temperature) and then increases again. The maximum population, however, decreases as the temperature of growth is raised, at least over the range investigated; hence the highest population density is not attained at the optimum temperature. Nevertheless it seems clear from the results given in Fig. 2 that temperature has little effect, over the range investigated, on the daily yield, which is a function of both growth-rate and population. In

other words, as the temperature is increased towards the optimum the lowered maximum population is compensated by the increased growth-rate.

Altering the temperature does, however, appear to affect the range of population density over which the maximum daily yield can be obtained. This is no doubt due to the effect of temperature on the maximum population and the fact that as the growth temperature is raised the maximum is reached more quickly. It means that, as the temperature is increased, the time of harvesting becomes more critical.

ACKNOWLEDGEMENT

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

The experiments were carried out by Mr. B. S. Pettit. The carbohydrate analyses were made by Mr. P. A. Lowe of the Royal Technical College, Salford, while visiting the Low Temperature Research Station as a vacation student in receipt of a D.S.I.R. Maintenance Grant.

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The Effects of 2:4-Dichlorophenoxyacetic Acid, 2:3:5-Tri-Iodobenzoic Acid, and Thiourea on the Vegetative and Reproductive Growth of *Avena sativa*

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Received 10 February 1956

SUMMARY

The effects of 2:4-dichlorophenoxyacetic acid (2:4-D), 2:3:5-tri-iodobenzoic acid (TIBA), and thiourea on the growth of *Avena sativa* have been studied in relation to subsequent leaf development, tiller production, and apical growth of the seedlings. Treatments with 2:4-D and TIBA depressed the emergence of each successive shooting leaf, but thiourea, at the concentration used, proved to be lethal. Subsequent observations on the tillering capacity of the seedlings show that treatment with 2:4-D significantly increases the rate of tiller production, but that TIBA fails to do so.

Longitudinal sections through the apices of the treated seedlings show that both treatments have inhibited the development of the apex. 2:4-D inhibition is the greater. In the concentrations applied, neither treatment caused any obvious abnormalities in respect of mature leaves.

INTRODUCTION

PLANT growth substances are known to produce abnormalities in cereal crops when applied as selective weed killers. Such abnormalities have been reported and discussed by a number of investigators including Large and Weston (1951), Andersen (1952 and 1954), and Audus (1953). Abnormalities are recognized by the occurrence of tubular leaves and trapped ears. In oats sprayed with 2:4-D when the plants were in the 2-leaf stage, dark-green rush-like growth developed. The effects were greatest with application at the 3-4-leaf stage. The period during which oat plants are susceptible to injury by 2:4-D is much longer than in wheat or barley; it extends from the early seedling stages to the middle of stem extension.

Andersen (1954) reports that following a spraying with 2:4-D, the most common abnormality in the meristematic regions of the barley shoot is a ring-like formation. Three weeks after spraying these rings begin to differentiate and to develop spikelets, but differentiation is irregular. Comparatively little work, however, has been carried out to study the effects of plant growth substances on the morphogenesis and organization of the primordial shoot apex in cereals. Such effects have been extensively investigated in other

plants. Waard and Roodenburg (1948), Gorter (1949 and 1951), and Wardlaw (1953) among others have dealt with modifications of the vegetative apex of tomato caused by application of TIBA. In view of the abnormalities found, it becomes increasingly important to study the effects of plant-growth substances on the growth, organ formation, and differentiation of tissues in the meristematic regions of cereal primordia.

EXPERIMENTAL METHODS AND MATERIALS

The experiment was conducted on Victory oats (*Avena sativa* L.). The seeds were germinated for 3 days at 20° C. and then planted in pots containing John Innes No. 1 potting compost.

When the seedlings had developed their first mature leaf and its corresponding shooting leaf, the former was cut half-way down its blade and the stump introduced into a small vial containing 1 ml. of the solution to be tested (Leopold, 1949). The solutions were taken up within 14 days of the commencement of treatment. The following were used: 2:4-D (sodium salt) and TIBA 500 mg./l., thiourea 1,000 mg./l. Controls were treated with distilled water. Each treatment was replicated 4 times, each replication consisting of 10 seedlings in one pot.

The experiment, which was conducted under conditions of short days and comparatively low temperatures, was terminated when the fifth leaf was fully developed. The apices were then dissected out, examined, and finally sectioned according to the technique of Sharman (1943 *a* and *b*).

EXPERIMENTAL RESULTS

I. *Macroscopic features.* The lengths of each of the successive shooting leaves were ascertained periodically as the leaves emerged. Measurements in cm. were taken from the basal node of each seedling to the tip of the leaf. Statistics were computed on the mean lengths per pot and the results are summarized in Table I.

TABLE I

Mean length of successive shooting leaves (cm.)

	Number of shooting leaf				
	2nd	3rd	4th	5th	6th
Distilled water (control)	13.8	23.1	23.6	28.7	34.7
TIBA	12.4	13.7	17.7	22.6	24.8
2:4-D	14.1	15.9	16.7	23.1	25.5
Thiourea	13.9	—	—	—	—
Variance ratio (F)	2.5	70.0	5.6	3.3	11.1
Probability	0.2-0.05	<0.001	0.05-0.01	0.20-0.05	0.01-0.001
Significant Difference (P 0.05)	1.7	0.7	5.4	6.7	5.6

The means for the second shooting leaf were ascertained from measurements taken 9 days after the treatments began. No significant differences are in evidence. Eleven days later, the effects of thiourea had proved to be lethal.

This treatment was included in the experiment in view of its effect, reported by Denny (1926), in stimulating a greater number of buds from a single eye of the potato. Thus its supposedly anti-auxin effects might compare favourably with similar effects reported with TIBA (Leopold, 1949). Further work with this substance is being undertaken.

Treatments with both TIBA and 2:4-D have an inhibitory effect on the emergence of each successive shooting leaf with the exception of the second. Differences between the means of the third, fourth, and sixth leaves reach

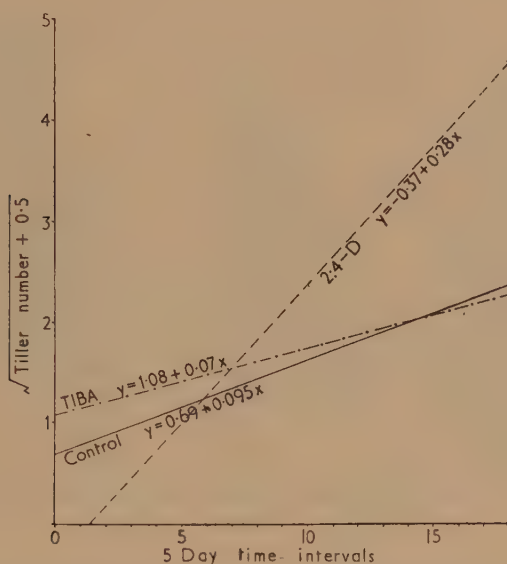


FIG. 1. Regression lines of tiller production in *Avena sativa* following treatments with 2:4-D, TIBA, and distilled water (control).

significance and the same trend is exhibited in the fifth leaf although the differences here fail to reach significance. No morphological disturbances such as tubular or rush-like leaves were produced.

Tillering commenced 26 days after the treatment was begun. Tiller numbers were recorded daily. The results were graphed at intervals of 5 days, the tiller counts, having been transformed according to the formula $\sqrt{(x+0.5)}$, x being the observed number (Snedecor, 1946). The regression lines, calculated for each treatment, are shown in Fig. 1 together with the coefficients for each. Treatment with 2:4-D significantly increases the rate of tiller production compared with the control, a 't' value of 14.2 for this comparison, reaching a very small probability. The rate fails to be significantly different following treatments with TIBA (a 't' value of 1.6 reaches a probability of 0.1), although tillering actually commenced 25 days earlier than with the 2:4-D treatment, and 35 days earlier than in the controls.

II. *Microscopic features.* The apices were examined under a dissecting microscope before they were embedded in paraffin wax. There was no evidence in the 40 apices of each treatment of any obvious abnormalities comparable with the ring-like formation in barley treated with 2:4-D, reported by Andersen (1954) or with the results of Waard and Roodenburg (1948). It was noticeable, however, that treatment with 2:4-D had inhibited the development of the apex to a remarkable extent. A trend in the same direction, but of a smaller magnitude, follows treatment with TIBA.

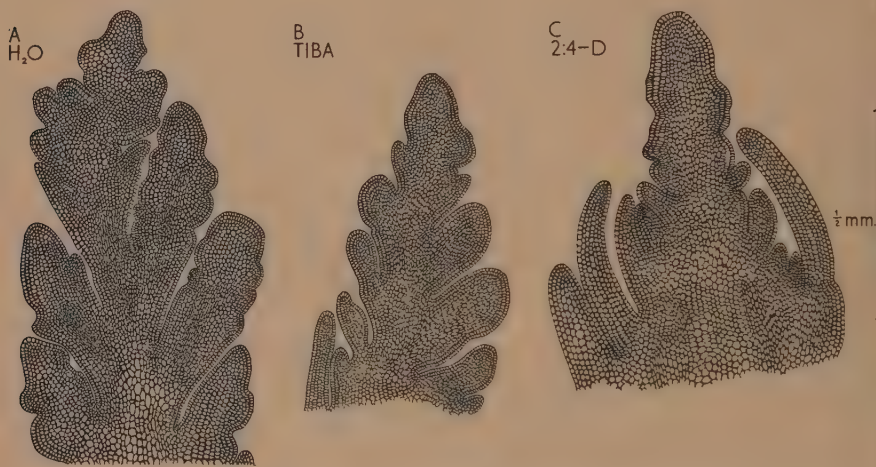


FIG. 2. Central longitudinal sections ($\times 220$) through the apices of *Avena sativa* treated with 2:4-D, TIBA, and distilled water (control).

Longitudinal sections through the plane of the youngest leaf primordium illustrate the extent of the inhibition in the 2:4-D and TIBA treatments compared with the controls. Fig. 2 shows a section of one typical apex from each treatment drawn by means of a camera lucida to a magnification of 220.

Bonnett (1937), in his studies on the morphological differentiation of the oat panicle, concludes that there are two phases in the development. The first phase is characterized by a short growing point and differentiating leaf initials. Then follows a marked elongation of the apex initiating the second phase. This is accompanied by the development of single, lateral alternate projections—the primordia of the branches of the first order. As these increase in size, branch primordia of the second and third order appear. Relating these conclusions to the present investigation, the apices derived from the control series show that elongation has been accompanied by the development of the primordia of the first and, in some cases, the second branches together with the initiation of the spikelets. The TIBA series shows less elongation of the apex and the development of the initials of only the first branches. The differentiation of the apex following treatment with 2:4-D is even more inhibited, being only in its first stages of panicle development.

There does not appear to be any fundamental modifications in the internal anatomy of the apex following treatment with either 2:4-D or TIBA at these particular stages in their development. Further investigations are being undertaken to study this aspect of the work in greater detail.

DISCUSSION AND CONCLUSIONS

It is apparent that 2:4-D applied in solution through the first leaf blade of young oat seedlings does not produce any of the abnormalities commonly associated with this substance when applied as a spray to the foliage leaves. This differential response can be partly attributed either to the concentration of the active substance in solution, or to the method of application. It is not easy to judge the effectiveness of the concentrations when methods of application differ. Andersen (1952) sprayed a small experimental plot of oats with 4 kg. per hectare 'in order to secure a fairly large number of abnormalities'. The above investigation shows that relatively weak concentrations of 2:4-D produce an overall inhibition of the apical meristem without any apparent abnormalities. This inhibition is accompanied by a very significant increase in the rate of tillering. Tillering is affected by a number of factors such as nutrition, soil moisture, seed size, day length, temperature, and depth of planting (Gardner, 1942). It is now widely known that it is also governed by apical dominance which could be related to the auxin content of the apex. Therefore a substance which is capable of opposing the action of auxin tends to increase tillering capacity. TIBA has been shown by Galston (1947) to oppose auxin action in this way. In the above experiment it is significant that it is 2:4-D which increases the tillering rate and not TIBA, but both treatments inhibit the growth of the apical meristem.

ACKNOWLEDGEMENTS

I am grateful to Professor R. Alun Roberts for the facilities he has placed at my disposal. I also acknowledge his continued interest in the work. I should also like to thank Mr. P. Greig-Smith, M.A., of the Department of Botany of this College for his assistance in the statistical analyses of the results.

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Some Aspects of Translocation in Root Nodule Plants

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Received 22 September 1955

SUMMARY

By the use of ^{15}N it is shown that the removal, over a short zone at the base of the shoot of a typical root nodule plant (alder), of the tissues external to the xylem does not interfere with the upward movement of fixed nitrogen from the nodules into the shoot. It is concluded that the fixed nitrogen, which is probably in organic form, can be translocated in the xylem in the transpiration stream, and that this is most likely its normal route. It is also shown that in unringed plants substantial enrichment in fixed ^{15}N is detectable in the shoot within 6 hours from the commencement of the exposure of the nodules to excess free ^{15}N . Structural and experimental evidence shows that the alder nodule is not a water-absorbing organ, and the mechanism of transfer of fixed nitrogen from the nodule into the transpiration stream is not obvious.

INTRODUCTION

IT is probable that within a plant bearing nitrogen-fixing root nodules there is, throughout the growing season, a two-way flow of organic matter along the plant axis. Products of photosynthesis must be conveyed from the leaves to the roots and nodules (the latter being characterized by relatively rapid respiration (Bond and MacConnell, 1955)), and products of nitrogen fixation from nodules to other parts of the plant. The evidence suggests that, at least in legumes, the fixed nitrogen is translocated in organic form. Thus Zelitch, Wilson, and Burris (1952) in attached nodules of soya bean, and Aprison, Magee, and Burris (1954) in detached nodules of the same legume, found that after relatively short exposure of the nodules to excess free ^{15}N the bulk of the fixed isotope in the nodules was present in the form of amino-acids, especially glutamic acid.

In the present work the effect has been tested of ringing the base of the stem on the movement of fixed isotopic nitrogen from the nodules into the shoot using alder (*Alnus glutinosa* (L.) Gaertn.). There is good evidence that the nodules of this plant have the same physiological significance and relation to the rest of the plant as those of legumes (Bond, Fletcher, and Ferguson, 1954; Quispel, 1954; Bond, 1955), and for ringing purposes alder has the advantage over many legumes that the stem becomes woody at an early stage and is readily ringed; moreover there are no complications arising from the presence of the cortical bundles characterizing some legumes. In addition to the experiments involving ringing, observations have been made on the

rapidity of the movement of fixed nitrogen into the shoot, and consideration given to translocatory events in the nodules themselves.

METHODS

The nodulated alder plants employed had been grown in water culture without combined nitrogen by methods already described (Ferguson and Bond, 1953), and were approximately 6 months old and fairly uniform in size when used. For the purpose of exposure to excess ^{15}N the root systems were sealed into jars of capacity 115 ml. partly filled with culture solution, leaving a gas space of 25 ml. in which most of the nodules lay. By methods previously described (Bond, 1955) this gas space was charged at atmospheric pressure with a gas mixture consisting of nitrogen (with excess ^{15}N), oxygen, and in some cases argon, the precise composition in each experiment being indicated later.



FIG. 1. Alder plant as used in the experiments, showing the position of the ring (r). ($\times \frac{1}{8}$)

Certain plants were ringed at the base of the stem prior to the exposure of their root systems to the isotope (Fig. 1). The ring was about 5 mm. in height and sufficiently deep to remove all the tissues external to the xylem, as was confirmed by the examination of sections under the microscope.

After the desired period of exposure, the shoot and root systems were separated at the ring or at a corresponding level in unringed plants, and the two parts, or in some cases the shoot and nodules only, prepared for mass spectrometric analysis as previously described (Bond and Scott, 1955). The analyses were again kindly carried out by Dr. R. I. Reed of the Department of Chemistry of this University. Control samples prepared from plant material not exposed to excess ^{15}N were included in those sent for analysis, as a check against instrument and other errors, while, as a further measure of control, samples of atmospheric nitrogen were also analysed. The results of these various control tests were satisfactory. In the course of 3 years over 50 control samples of the author's plant material have been analysed, a recent review of the results showing the mean ^{15}N content to be 0.372 atom per cent., which is satisfactorily close to the accepted value. This mean figure has been used in the next section in calculating increases in ^{15}N content shown in alder plants as a result of experimental treatment. The standard deviation of the above control values was 0.012 atom per cent., which provides a measure of the variability of the instrument.

EXPERIMENTAL DATA

The results of the first ringing experiment are presented in Table I, Series I. Previous experience has shown that when the root system of a nodulated alder

plant is exposed to excess ^{15}N for several days and the different parts of the plant then analysed, the highest level of fixed ^{15}N on the atom per cent. basis is found in the nodules, though some is also present in the roots and shoot due to translocation from the nodules (Bond, 1955). The data for the unringed plants in Table I, Series I, show a similar picture, allowing for the fact that here the nodules and roots were analysed together. The extent of enrichment, which is a measure of fixation per unit of total plant nitrogen, is unusually low in plant 1, due undoubtedly to some fault in the experimental procedure, though this could not be traced. But the distribution of the fixed nitrogen between the root system and the shoot is approximately similar in all three plants, and on the average the increase in concentration of ^{15}N in the total nitrogen of the shoot was, by the end of the experimental period, half that in the root system.

TABLE I

Effect of stem-ringing on translocation of fixed nitrogen into shoots

The mean shoot height of the *Alnus* plants employed was 18 cm. in Series I and 14 cm. in Series II. The gas space in the jars enclosing the root systems was charged with a mixture initially 30% oxygen and 70% nitrogen with 20 atom % ^{15}N . The root systems remained exposed to this for a period of 5 days under normal greenhouse conditions in early September in Series I but plants were held in a ventilated dark cabinet at 21° C. in mid-September in Series II.

Plant number	Treatment	Increase in ¹⁵ N content, as atom % of total nitrogen, over normal value (0.372)		Ratio,
		Root system	Shoot	<u>Shoot increase</u> Root increase
Series I				
1	Not ringed	0.088	0.039	0.4
2	” ”	0.218	0.138	0.6
3	” ”	0.334	0.192	0.6
Means		0.213	0.123	0.5
4	Ringed	0.085	0.090	1.1
5	”	0.103	0.098	1.0
6	”	0.091	0.063	0.7
Means		0.093	0.084	0.9
Series II				
1	Not ringed	0.095	0.111	1.2
2	” ”	0.100	0.065	0.7
3	” ”	0.055	0.015	0.3
Means		0.083	0.064	0.7
4	Ringed	0.067	0.024	0.4
5	”	0.082	0.019	0.2
6	”	0.072	0.020	0.3
Means		0.074	0.021	0.3

The fixation in the ringed plants per unit of total plant nitrogen was clearly substantially reduced in comparison with the unringed plants, no doubt as

a result of the cessation of the supply of carbohydrates to the nodules from the leaves (Lindstrom, Newton, and Wilson, 1952; Virtanen, Moisio, and Burris, 1955). The difference would have been greater had the fixation in one of the unringed plants not been unusually low, as noted. But it is clear that the interruption of the phloem did not interfere with the upward movement of fixed nitrogen into the shoot; actually the figures suggest that a somewhat higher proportion of the fixed isotope migrated into the shoot in these ringed plants, perhaps because the migration continued after fixation had been reduced to a low level. The absence of any visual signs of nitrogen deficiency in the leaves of the ringed plants at the end of the 5-day period is in keeping with the isotopic evidence that fixed nitrogen continued to reach the shoots.

Since the total nitrogen contents of the root systems and shoots of the plants to which Table I, Series I, refers are known, it has been possible to calculate the absolute increases in ^{15}N content. For the unringed plants the mean increase for the root system was 15 $\mu\text{g.}$ and for the shoot 20 $\mu\text{g.}$, the corresponding figures for the ringed plants being 7 $\mu\text{g.}$ and 21 $\mu\text{g.}$ These absolute figures make it still more evident that a greater proportion of the fixed ^{15}N was located in the shoot of the ringed plants by the end of the experimental period than in the case of the unringed plants, as discussed above. These figures also indicate a mean fixation of 35 $\mu\text{g. }^{15}\text{N}$ per unringed plant and 28 $\mu\text{g.}$ per ringed plant, but this comparison is affected by the abnormally low fixation shown by one of the unringed plants and the relatively small size of another. It is clear, however, that the presumptive isolation of the nodules from the normal source of carbohydrates resulting from ringing by no means extinguishes fixation, suggesting that reserves are available in the nodules. It should be noted that the total fixation, i.e. including ^{14}N , was much greater than of ^{15}N alone but cannot be calculated because the proportion of ^{15}N in the nitrogen that is being fixed in the nodules falls rather rapidly with the present experimental arrangement (Bond, 1955).

In a second experiment, carried out before the results of the first became available, the plants were kept in darkness during the whole period of exposure to excess ^{15}N . This was to anticipate a possible objection to the first experiment, namely that had the ringing in that case stopped the movement of fixed nitrogen into the shoot it might have been argued that this was not because the required channel had been interrupted, but merely the result of the curtailment of fixation occasioned by ringing, so reducing the 'head' of fixed isotope in the root system. It seemed probable that by darkening the plants the disparity in extent of fixation between unringed and ringed plants could be reduced. As already seen, a cessation of translocation of fixed nitrogen following ringing was not shown in the first experiment, so that the second one loses some of its point; the results serve, however, to confirm those of the first, and are presented in Table I, Series II. Fixation per unit of total plant nitrogen was clearly substantially reduced in the unringed plants as compared with the corresponding plants of the first experiment, which may be an effect of darkening. In the ringed plants fixation was still further reduced, but all three

shoots show slight increases in ^{15}N content which are probably significant considered collectively. Ringing might appear to have impeded somewhat the movement of nitrogen into the shoot, but at these very low levels of enrichment and with rather few plants too much weight cannot be placed on the data. The mean absolute gains of ^{15}N in this experiment were 15 $\mu\text{g.}$ per unringed plant and 10 $\mu\text{g.}$ per ringed plant.

TABLE II

Test for translocation of fixed ^{15}N into shoot within 6 hours of the commencement of fixation of excess ^{15}N

The *Alnus* plants (all unringed) employed were similar in size to those used in the ringing experiments. Their root systems were exposed to a gas mixture comprising 20% oxygen, 10% nitrogen (with 36 atom % ^{15}N), and 70% argon. The experiment was carried out under daytime greenhouse conditions in mid-August.

Plant number	Increase in ^{15}N content, as atom % of total nitrogen, over normal value (0.372)	
	Nodules	Shoot
1	0.222	0.097
2	0.135	0.037
3	0.445	0.121

In a different type of experiment the shoots of unringed alder plants were examined for ^{15}N content after relatively short exposures of the nodulated root systems to excess free ^{15}N . The results of an experiment carried out in mid-August in which a 6-hour exposure was given are presented in Table II. The shoots of all three plants employed show a substantial increase in ^{15}N content which is roughly proportional to the enrichment indicated for the nodules. Clearly a period of 6 hours suffices for the fixation and translocation into the shoot of substantial amounts of nitrogen. On a later occasion the shoots of six plants became available, the root systems of which had been exposed for 3 hours (in mid-September) to a gas mixture similar to that specified in Table II. Here, however, the activity of the nodules in fixation was much smaller than might have been anticipated from the 6-hour experiment, since the mean enrichment shown by the nodules was only 0.024 atom per cent. This is no doubt due to a seasonal effect; it is not surprising that none of the six shoots showed a significant increase in ^{15}N content. It seems very probable that a demonstrable translocation of nitrogen into the shoot would be obtained after 3 hours' exposure at a season when the nodules were more active.

DISCUSSION

The results of the ringing experiments show that in alder the interruption at the base of the shoot of the tissues external to the xylem does not interfere with the upward movement of fixed nitrogen from the nodules into the shoot. It is therefore concluded that the fixed nitrogen, which as noted in the

Introduction is probably in organic form, can be translocated through the xylem in the transpiration stream. It seems almost certain that this is its normal route, and that it does not travel via the phloem.

At the season of greatest activity (August) several milligrams of fixed nitrogen must pass each day into the shoot of a first-year alder plant, and much larger amounts in the case of second-year plants which were also available, and the possibility of a direct examination of xylem sap for fixed nitrogen has been considered. Unfortunately the plants show no root exudation during summer

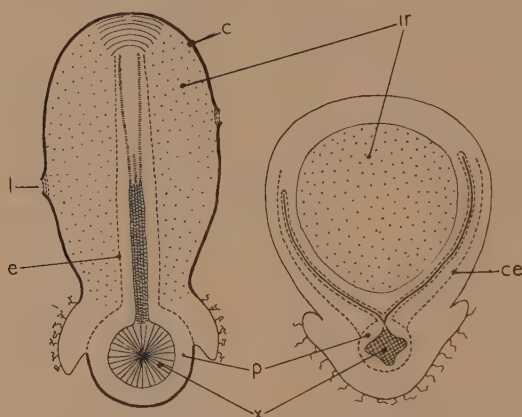


FIG. 2. Diagrammatic representations of Alder (left) and Broad Bean (*Vicia faba* L.) nodules as seen in sections cut transversely to the root. x, xylem; p, phloem; e, endodermis; c, cork; ir, infected region; l, lenticel; ce, common endodermis. (Alder $\times 13$, Broad Bean $\times 27$.)

months, while negligible amounts of sap have been obtained from the wood by the centrifugal or pressure methods. Wolfgang and Mothes (1953) report very briefly that relatively large amounts of citrulline were detected in sap bled from field trees of alder, presumably in the early spring, but the immediate origin of this amino-acid is a matter for conjecture.

It is clear from earlier work that in soya bean plants, some 80 to 90 per cent. of the fixed nitrogen is steadily exported from the nodules (Bond, 1936). Jensen (1948) and Virtanen (1952) reported essentially similar findings for other legumes. The evidence now presented that substantial enrichment in fixed ^{15}N is detectable in shoots within 6 hours of the commencement of the exposure of the nodules to excess free ^{15}N is consistent with this earlier work.

Certain aspects of nodule structure are relevant in considerations of translocation. As indicated in Fig. 2 the general structure of an alder nodule is more akin to that of a normal root than is the case with the legume type, exemplified by broad bean. In alder a central stele is present, the endophyte being resident in the hypertrophied cortical cells. The endodermis enclosing the stele is in the secondary (suberized) condition in the basal half of the nodule, and if this suberized layer is credited with the effect usually assigned

to such a layer then exchange of materials between stele and cortex must be mainly confined to the apical region. A similar limitation may be imposed in the legume nodule by the presence of an endodermal sheath, secondary in the basal part of the nodule, round each of the numerous vascular strands. A notable feature of alder and some other non-legume nodules is that a superficial cork layer develops at an early stage, and in alder the nodule is completely enclosed by this layer a few weeks after the origination of the nodule, except that lenticels are present. To some extent the common endodermis of the legume nodule (Frazer, 1942) seems to be comparable functionally with the superficial cork of alder, but it does not extend over the apex of the nodule.

Thus structural considerations suggest that, except possibly in a very early stage of development, the alder nodule is not a water-absorbing organ. Experimental confirmation of this was sought in experiments rather similar to those carried out in the present author's laboratory by Frazer (loc. cit.) in a study of endodermal effects in legume nodules. Young alder plants were placed with their root systems in 0.005 per cent. aqueous solutions of methylene blue or neutral red for 48 to 72 hours, and then by means of sections the ingress of the dyes into the roots and nodules was studied. Roots showed deep staining in root hairs, cortex, some xylem vessels, and in the root-tip region. In nodules, apart from a few instances of very local penetration to a depth of 2 to 3 cells only, there was in general no penetration past the cork layer. Expectations based on structure are thus confirmed.

Since water absorption from the external medium by alder nodules seems improbable the mechanism of transfer of fixed nitrogen from the nodules to the transpiration stream must at present remain a matter of conjecture. In the case of the legume nodule the possibility of water absorption from the environment exists.

The present experiments throw no light on the movement of fixed nitrogen from the nodules to the distal parts of the roots. Possibly the fixed nitrogen is redistributed to such regions from the leaves, via the phloem.

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The Concept of Minimum Leaf Number

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Received 5 December 1955

SUMMARY

Several quotations of minimum leaf numbers for floral initiation are given in the literature, but it was thought desirable to obtain further evidence that they do represent, as claimed by Purvis and Gregory for rye, obligatory vegetative growth preceding the attainment of Klebs's *Blühreife*. The effects of mineral starvation on leaf number, as in Purvis's original experiments, were therefore tested with *Xanthium*, *Eupatorium adenophorum*, and *Celosia argentea*. In each of these, near-constant leaf numbers are characteristic of plants induced to flower rapidly by continuous short-day treatment. In each, treatments such as starvation which retard vegetative growth do somewhat reduce the numbers. In *Xanthium* indeed, the concept of *Blühreife* is superfluous, for the 8 leaves to be accounted for could be explained as the primordia present in the seed plus the extra leaves initiated during daylength induction. For the other two plants, on the other hand, the sum of these is still many short of the required number, and for them a concept of *Blühreife* expressed by leaf number is retained, but with the admitted doubt whether all factors controlling their flowering are yet known.

KLEBS in 1913 invented the term '*Blühreife*' for a postulated stage in the development of a plant signifying its 'ripeness' for environmental conditions to induce flowering. It is evident that he could not himself characterize the stage, save by the negative criterion that none of the environmental variants at his command would induce initiation in rosettes of *Sempervivum funkii* before the end of the summer.

Gregory and Purvis (e.g. Purvis, 1934) borrowed the term 'Ripeness-to-flower' from Klebs, and gave it a quantitative meaning in terms of leaf number. In a winter rye they could discover no daylength or vernalization treatment which would reduce the number of leaf initials on the main axis, preceding the ear, below that typical of the corresponding spring variety, i.e. seven. It seemed unlikely that the correspondence was coincidental and they suggested that the first seven primordia differentiated from the apex must, in some fundamental way, be different from the rest and inherently incapable of producing spikelets in their axils. It was concluded that in both winter and spring varieties of rye 'ripeness-to-flower' is equivalent to the completion of seven leaf primordia, and that in other plants too a specific leaf number would indicate the attainment of this condition.

This is an attractive hypothesis and is widely quoted as a general principle, minimum leaf numbers having been quoted for some other plants, e.g. for wheat by McKinney and Sando (1933), for mustard and barley by von Denffer (1939), for henbane by Lang and Melchers (1943), for tomato by Waard and

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Roodenburg (1948) and for certain varieties of peas by Barber and Paton (1952) and by Leopold and Guernsey (1953). In none of these cases, however, can the data presented be regarded as strict evidence that the number quoted is, indeed, an immutable minimum.

It was therefore considered desirable to determine leaf numbers in experiments in which a deliberate attempt was made to change them. As a beginning *Xanthium* (a plant whose flowering behaviour is otherwise well known) was chosen for the tests, and through the help of W. W. Schwabe seed was obtained of the stock used by Hamner in his long series of photoperiodic investigations. Hamner himself appears to believe (e.g. 1944, p. 579, and 1948, p. 106) that this plant has a stage of ripeness-to-flower but does not quote any specific leaf number.

The general scheme of the experiments here described was to attempt to grow plants of as wide a range of size, i.e. rate of growth, as possible by manipulating their mineral nutrition, whilst exposing them to photoperiodic conditions judged most favourable to earliest initiation of flowers. Records of the final leaf number attained before the terminal inflorescence would then be a test of the constancy of leaf number. This is, of course, exactly parallel to the tests made by Purvis on rye, but hers were necessarily incomplete because at the time (1934) the most effective vernalization methods for that plant had not been realized.

EXPERIMENTAL

1. *Xanthium* seeds were sown in sand in glass pots. Until germination occurred the pots were all kept watered with distilled water and exposed to $16\frac{1}{2}$ -hour days. Germination, however, was very sporadic (see 3 below) and it was decided to begin treatments as seedlings became available and to complete one replicate before the second was started.

The plants were then thinned to one per pot; and there were 64 pots in all, i.e. high *v.* low nitrogen, high *v.* low potassium, high *v.* low magnesium, intact *v.* half defoliated, $16\frac{1}{2}$ -hour *v.* 8-hour daylengths, all times two replications.

The complete 'high' level solution had the composition: 0.304 g. NaNO_3 , 0.0303 g. KCl , 0.0417 g. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0546 g. $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 0.222 g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.046 g. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.0024 g. Fe-tartrate in 200 cc. solution per pot. The 'low' nutrient level was, in each case, one-tenth that of the high dosage.

The daylength treatments were given in a cool greenhouse, thermostatically frost protected at 10°C . Natural light was used as far as possible but supplemented by electric light to bring the total always to $16\frac{1}{2}$ hours for the long daylength. For the short days, the plants were transferred between 4 p.m. and 8 a.m. to a light-tight annexe provided with forced air circulation from the main greenhouse.

The defoliation treatment consisted in removing every alternate leaf as soon as it was distinct from the terminal bud.

It soon became evident that the concentration of salts was too high, at least in the high nitrogen series and several of these plants were injured. The long-

day part of the experiment was, in fact, not completed, although sufficient plants survived to show that *Xanthium* will eventually flower normally in days as long as $16\frac{1}{2}$ hours.

The plants were harvested individually on the day their first male florets opened. As an estimate of plant size, the tops and roots free of sand, were weighed after drying at 80°C . for 48 hours. Data from the short-day part of this experiment are collected in Table I.

2. The scheme of manurial treatments was altered considerably for the second trial: instead of factorial combinations, the treatments were dilutions of a complete culture solution, i.e. of the full 'high'-level solution used in Expt. 1, but applied a quarter at a time at fortnightly intervals. The dilutions were the same at one-quarter, at one-sixteenth, and at zero strength. The 'zero' concentration consisted only in watering with tap water in contrast to the others for which distilled water was used. As before, each manurial treatment was combined with long ($16\frac{1}{2}$ hr.) and short (8 hr.) daylengths, but the defoliation treatment was omitted. For each combination there were 8 replicate pots, one plant to each. Germination, as in Expt. 1, was spread over a considerable period, but early growth was otherwise satisfactory. Again, however, few of the long-day plants survived long enough to reach anthesis (Table I).

3. This was essentially a repeat of Expt. 2, except that a special attempt was made to complete the long-day half. As before the main part of the experiment was a sand-culture trial in which the full, one-quarter, one-sixteenth, and nil concentrations of the complete culture solution as used in Expt. 1 were applied; in each case as quarter doses applied fortnightly. The full-strength solution was also applied to plants from which the cotyledons were removed as soon as they expanded. The low nutrient-level treatments were this time, however, only attempted in the short-day series and instead, in both daylengths, two extra series were included: rich potting soil in 6-inch earthenware pots, and full-concentration sand culture in which the plants were potted on into larger glass pots as they grew—the volume of sand and solution being adjusted on each occasion. It had been discovered in the meantime that regular germination of *Xanthium* seeds can be obtained if the testas are removed. Uniform germination for this experiment was therefore assured by soaking the seed, removing the testas, and allowing germination overnight in an incubator. The seedlings were then ready for transfer to the pots next day and the daylength and manurial treatments could be started at once.

There were 8 plants in each treatment.

Good growth was maintained in all treatments until harvest and it was therefore worth while keeping fuller records than were attempted in Expts. 1 and 2 (Table II).

These three experiments showed reasonably well that the leaf number normally produced by *Xanthium* in continuous short days is not, after all, unchangeable and can be reduced if sufficiently stringent starvation conditions

TABLE I
Xanthium. Expts. 1 and 2

Expt. 1	Leaves excl. cotyledons on main axis before terminal inflorescence			Average total dry wt. (g.)
Low N v. high N . . .	9.9	9.4	NS	1.13:0.85 S
Low K v. high K . . .	9.5	9.8	"	0.88:1.10 NS
Low Mg. v. high Mg. . .	9.8	9.5	"	0.80:1.17 S
Defoliated v. intact . . .	9.4	9.9	"	0.82:1.16 S
Expt. 2				
Full dosage of nutrients . . .	7.63	(range 6-9)		0.788
One-fourth dosage . . .	7.88	(" 6-10)		0.437
One-sixteenth dosage . . .	6.75	(" 5-8)		0.200
No added nutrient . . .	5.75	(" 4-7)		0.086
S.D. at P 0.05 = 0.99			S.D. at P 0.05 = 0.164	

TABLE II
Xanthium. Expt. 3

Short day	Leaves excl. cotyledons on main axis before terminal inflorescence	Measures of maturity		
		Dry wts. (g.)	Total leaf area (cm. ²)	Height on day of antithesis (cm.)
1. Soil . . .	8.6 (range 8-10)	1.36	287.8	9.5
2. Sand: fully manured, repotted . . .	7.9 (" 7-9)	0.73	135.3	8.9
3. Sand: fully manured	8.3 (" 7-9)	0.54	106.1	7.7
4. Sand: one-fourth full dosage . . .	7.6 (" 7-8)	0.32	58.1	6.9
5. Sand: one-sixteenth	7.6 (" 7-8)	0.18	29.8	4.6
6. Sand: cotyledons excised . . .	7.0 (" 6-8)	0.18	29.8	3.1
7. Sand: no added nutrients . . .	6.9 (" 6-8)	0.09	12.1	3.2
Long day				
8. Soil . . .	31.3 (" 28-35)	30.5	1,694	122.3
9. Sand: fully manured, repotted . . .	32.6 (" 30-37)	8.52	485.0	44.2
(6)(7) < (1)(2)(3)(4)(5) $\overset{\text{SD at}}{P 0.05 = 0.06}$ (7) < (6)(5)(4)(2) < (1) < (9) < (8) $P 0.05$				

are imposed. The meaning of this is discussed at length below, but it will be realized that with only 8 or 9 leaves as the norm, *Xanthium* does not offer much latitude for a convincing reduction of leaf number by these methods, and therefore a further test on the same plan was carried out with *Eupatorium adenophorum*, also a short-day plant.

It was demonstrated in preliminary trials that this species of *Eupatorium* flowers when exposed to daylengths of 8 hours and can, on the other hand, be held vegetative indefinitely in 16½-hour days. With *Eupatorium*, unlike

Xanthium, the effects of short-day induction are not permanent, for plants in flower eventually revert to vegetative growth when transferred to long days. Since *Eupatorium* can behave as a perennial, the possibility of response to 'vernalization' was tested also. (Cf. Schwabe (1950) on *Chrysanthemum*.)

4. In a factorial design, daylength treatments, chilling and starvation treatments were combined as follows:

The daylength treatments were exactly as described for the *Xanthium* experiments. The chilled plants were exposed out of doors for 3 weeks to the natural frosty nights of the early spring. Otherwise the plants were protected against temperatures below 10° C. in the greenhouse described. These treatments were applied to seedlings raised from germination in sand, to others in rich potting soil, and also to plants raised from cuttings. The cuttings were derived from two sources—from a large plant raised and grown throughout in 16½-hour days in the greenhouse, and also from a large plant growing under natural conditions in the garden. Each combined treatment was applied to 4 plants and the whole experiment was thus 2 daylengths × 2 chilling treatments × 4 types of plant × 4 replications.

Rapid and uniform germination was secured by soaking the seed in water before sowing during exposure to light. The cuttings, each with three pairs of expanded leaves, were rooted in sand under bell-jars, after 12 hours treatment in IAA. They were all exposed to 16½-hour days until rooted (7 July), but the daylength treatments for the seedlings were begun immediately on germination (22 May). Exposure out of doors was begun on 7 July for both.

Before the chilling treatment was concluded, some of the cuttings from the garden stock were found to be budded, and after return to the greenhouse flowering became general in plants from that stock, independently of daylength and independently of exposure out of doors. Since (Table III) no vernalization effect was in fact demonstrated, this is an interesting example of daylength induction, for these cuttings had last been exposed to natural daylengths on 10 May. The plants of this stock which flowered in long days produced a few more flower trusses from axillary branches, but later vegetative shoots grew out, and the plants reverted to purely vegetative growth.

The behaviour of the remaining plants is summarized in Table III, but it should also be mentioned that the onset of flowering among the seedlings in sand was very irregular, the last plant not being visibly budded until 17 April the following year.

The plants were harvested in pairs. As soon as any anthesis occurred in a short-day plant, it and the corresponding long-day plant were harvested by severing below the lowest or the cotyledonary node. The middle section of the 4th internode (cf. Dormer 1951) was cut out with a pair of razor blades fixed 3 cm. apart, and this and the rest of the top were dried at 80° C. The dry weights used as a measure of plant size in Table III include the separated sections but are not comparable with the estimates of size used for *Xanthium* in the preceding tables as the roots are not included.

5. Seedlings of *Eupatorium* germinated in 16½-hour daylengths were

TABLE III
Eupatorium, Expt. 4

	Leaves excl. cotyledons on main axis to terminal fl. truss	Measures of maturity		
		Dry wt. tops (g.)	Linear density of 4th inter- node (g.)	Height on day of harvesting (cm.)
1. Seedlings in sand no add. nut. . .	32.3 (range 30-35)	1.02	0.117	22.8
2. Seedlings in soil . .	34.4 (" 33-37)	12.8	0.475	57.0
3. Cuttings from green- house stock . .	26.4 (" 24-27)	6.17	0.229	49.4
4. Cuttings from gar- den stock . .	19.9 (" 12-29)
(a) Chilled . .	31.0 (" 26-37)	6.37	0.259	42.7
(b) Not chilled . .	31.2 (" 24-35)	6.93	0.288	43.4
X Short day	5.98	0.239	41.9
Y Long day	7.32	0.306	44.2
	(2) > (1) P 0.05	(1)-(3)	(1)-(3)	(1)-(3)
	(2) > (3) P 0.001	SD = 2.13	SD = 63	SD = 3.80
	(a), (b) NS	(a), (b) NS	(a), (b) NS	(a), (b) NS
		X, Y NS	X < Y P 0.05	X, Y NS

TABLE IV

Eupatorium, Expt. 5. Leaves produced on the main axis to the terminal flower truss of the inflorescence

	On the cutting alone	Including stock
Series: 0	—	36.0 (range: 35-37)
1	31.0 (range: 31-31)	32.0 (" 32-32)
2	28.5 (" 28-29)	31.5 (" 31-32)
3	28.4 (" 26-28)	33.4 (" 31-33)
4	20.3 (" 19-23)	27.3 (" 26-30)
5	28.1 (" 25-31)	37.1 (" 34-40)
6	31.0 (" 30-32)	42.0 (" 41-43)
7	30.0 (" 26-34)	43.0 (" 39-47)

potted up, a pair into large (10 in.) and another pair into small (4 in.) pots of soil. The long-day treatment was continued until the plants were large enough for cuttings to be safely taken from them. One pot of each pair was then transferred to 8-hour daylengths. The remaining 2 plants were kept on in long days and used as a source of cuttings of successively greater maturity as represented by leaf number. On the first occasion the tops were severed just below the third node—not counting the cotyledonary node—and the piece removed used as a cutting. On successive occasions the axillary branches which grew out from the severed nodes were severed below their third nodes and used likewise. The cuttings were rooted under bell-jars after treatment with IAA as in Expt. 4 but short-day treatment was begun immediately. The successive lots as labelled in Table IV had thus the same number of expanded leaves when taken into short days but were each two nodes older

(4 leaves) measured by the previous history of the stock plant. As the stock plants became older, size difference due to the restriction of the smaller pot became more and more pronounced and a distinction could then be made between age and size of the stock plants which otherwise, of course, are correlated.

6. Seedlings of *Eupatorium* were germinated in boxes and grown in long days (17 January to 4 June). They were then potted up separately. By this time they were of about the same size as was reached by the seedlings of Expt. 4 when they came into flower, and to test that indeed they were past the stage of ripeness-to-flower, 5 of the smallest were transferred to short days. One appeared to be budded on 1 August, and as on dissection the rest of the five were also then found to have initiated inflorescences, the experiment proper was begun.

On that date the terminal buds of 4 plants were fixed for microtoming, and the rest (60) were transferred to 8-hour daylengths. After respectively 1, 2, 4, 8, 16, 24, and 32 days, sets of 4 plants were returned to long days, leaving 4 plants in short days permanently. (N.B. A 64-day set had also been planned; but in error 4 plants were moved back to long-days after only 9 days instead.) The rest were used to provide apices for sectioning, 4 being taken at a time, at weekly intervals from the beginning of the short-day treatment.

When anthesis had occurred in every plant induced to flower, the remainder were dissected. Data from this experiment will be found in Table V and reproductions of representative sections of the apex are shown in Fig. 1.

TABLE V

Eupatorium. Expt. 6

(a) Incidence of flowering (in four plants):

Induction period: 1 short day	nil
Induction period: 2 short days	nil
Induction period: 4 short days	nil
Induction period: 8 short days	nil
Induction period: 16 short days (loss of apical dominance in one)	
Induction period: 24 short days	3
Induction period: 32 short days	4
Continuous short-day treatment	4

(b) Number of leaves on main axis excluding cotyledons:

	To terminal truss	To beginning of inflorescence
24 short days	40.6	32.0
32 short days	42.0	33.0
Continuous	40.0	32.0

(Average number of leaves, including primordia at time of transfer to short days = 30.2)

(c) Average number of capitula in whole inflorescence:

24 short days	75.3
32 short days	127.5
Continuous	144.8

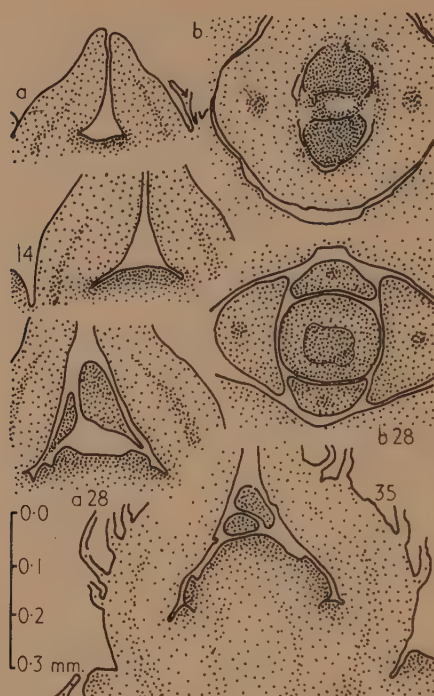


FIG. 1. *a* and *b*: L.S. and T.S. of an apex in long days; that is, vegetative.

14, 28, and 35: after 14, 28, and 35 days respectively, in short days. (In *b28* the ultimate tip section is superimposed on the next below the tip, to show the origin of the first flower-truss primordium.)

7. It had been hoped to continue experiments with *Eupatorium* at Achimota using the same seed stock. So far, however, no means of inducing flowering (e.g. by any daylength treatment) in this plant has been successful there. Vegetative growth is vigorous but shows the diageotropic tendency described by Schwabe (1950) as indicative of an unvernallized condition in chrysanthemums. This suggests that *Eupatorium* too may be vernalizable after all, i.e. at a relatively high temperature.

As a substitute for *Eupatorium*, *Celosia argentea* has been tried and in several ways has proved an ideal subject for this type of experiment. Thus the day length control of flowering is very clear cut: flowering occurs rapidly in 8-hour days, more slowly in the natural daylength, and the plants remain vegetative indefinitely in $16\frac{1}{2}$ -hour days. The normal leaf number in continuous short days is moderately high: about 15 or 16; and furthermore, as the beginning of the inflorescence is sharply distinguished from the leafy nodes of the stem, as in the cereal ear, the leaf numbers counted are more obviously analogous to those of rye than can be claimed for those of *Xanthium* or *Eupatorium*. The seeds are very small and even on germination only 4 leaf initials could be found additional to the cotyledons.

Seed from a single wild plant was germinated under 8-hour daylengths, in sand kept watered with tap water. The seedlings were transplanted singly into 32 glass pots each holding 2 Kg. of dry sand. Each pot received 800 ml. of its appropriate solution, the excess being retained in a saucer. The solutions were successive dilutions of the following, which was adapted from a recipe of Piper's (quoted in Hewitt, 1952), per litre: 1 g. KNO_3 , 0.5 g. KH_2PO_4 , 0.5 g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. As usual, 'no added nutrients' means watered with tap water only. There were 4 replicates of each nutrient level. 8-hour days were continued throughout the experiment.

One plant damped off, but fortunately not before its leaf number could be counted. With this exception, growth was excellent although it will be seen (Table VI) that the highest nutrient concentration was probably too high for maximum growth. Each plant was harvested on the day it first had any florets open. Data from this experiment are shown in Table VI.

TABLE VI
Celosia. Expt. 7

		Leaves excl. cotyledons on main axis before terminal inflorescence	Measures of maturity		
			Dry weight (g.)	Fresh weight (g.)	Leaf area (cm. ²)
a.	Full dosage of nutrients	15.50	3.12	30.6	105.0
b.	Half dosage	15.25	6.34	62.0	211.9
c.	Quarter dosage	14.50	5.55	51.5	172.3
d.	One-eighth	11.75	4.42	47.6	145.6
e.	One-sixteenth	15.00	2.74	30.8	80.9
f.	One-thirty-second	14.00	1.58	18.2	45.4
g.	One-sixty-fourth	11.50	0.85	7.35	19.2
h.	No added nutrients	6.75	0.22	2.10	6.8
SD 3.17			Dry wt. $abcde > g > h$		
			Fr. wt. $bcd > fgh$ and		
			$ae > g > h$		
			Lf. area $abcd > f > g > h$		
			all at P 0.05.		

DISCUSSION

All flowering plants appear to precede flower initiation by the production of leaf primordia. For photoperiodic or vernalizable varieties growing in nature, the duration (and, therefore, the amount) of this vegetative growth is determined by the chance of exposure to suitable environmental conditions for flowering. But not uniquely so, for given the most favourable conditions for flower induction from the very beginning of germination, some leafy nodes are invariably produced below the inflorescence, and moreover an initial period of vegetative growth is just as characteristic of those plants which appear to have no specific environmental requirements for initiation. The completion of this early vegetative growth is the attainment of the condition of ripeness-to-flower, in Klebs's sense of the word *Blühreife*. A

considerable diversity of ideas on the nature of the vegetative period and the reasons for its termination are implied in the literature. The following list is no doubt not exhaustive but includes those which can be criticized in relation to the experiments described above:

(A) That the vegetative phase is obligatory and of (a) a morphological nature consisting of (1) the completion of a specific number of leaf primordia (Gregory and Purvis—rye), (2) a specific degree of anatomical maturity (Dormer—bean); or (b) a physiological nature, consisting of the accumulation of a specific amount of (1) carbohydrate (Klebs—*Sempervivum*, &c.), (2) carbohydrate relative to nitrogen (Kraus and Kraybill—tomato), (3) size in an unspecified sense (bulbs, e.g. Heath and Holdsworth—onion).

(B) That the duration of the vegetative phase is a temporal accident governed by the relative rates of two types of growth, vegetative and reproductive, of which reproductive is always the slower because (a) photoperiodic or other floral induction takes time, (b) floral initiation is inhibited by some part of the seed, e.g. the cotyledons (Barber and Paton—peas).

(C) That the vegetative growth represents the unfolding of those parts already present in the embryo.

When *Xanthium* is grown in short days from germination, the number of leafy nodes on the main stem preceding the involucre of the terminal male inflorescence has always been about 8 or 9 in addition to the cotyledons for well-grown plants. This has been found so (cf. Expts. 1, 2, and 3) at different times of the year, i.e. independently of the temperature and the time taken to reach anthesis. In a small experiment not reported above, the same modal leaf number was found if naked embryos in petri-dishes were exposed to short days from the very beginning of germination. Similarly in Expt. 1 no real evidence of altered leaf numbers was found over an eightfold range of plant size (as measured by dry weight) induced by mineral starvation or by defoliation. It is true that the leaf numbers ranged from 8 to 12 over the whole experiment, but the extremes were not associated with particular treatments and the variation may well have been of genetic origin. So far then there is reason to subscribe to the general validity of Gregory's concept of minimum leaf number (A a 1). However, when the second and third experiments are also considered, experiments in which the nutrient starvation imposed at the lowest levels was much more severe, there is real evidence of a reduction in leaf number by starvation, even though the magnitude of the reduction was less than 2 leaves on the average in Expt. 2 and hardly more than one in Expt. 3. It will be noticed in both cases that reduction is only apparent at the lowest nutrient level and it is therefore possible that the starvation treatments applied by Purvis to rye were, like those of Expt. 1, not sufficiently drastic, indeed the 'low' level of one-tenth the standard dosage was adopted in Expt. 1, in imitation of her treatments.

For *Eupatorium* and *Celosia* too a significant reduction of leaf number was produced by extreme nutrient starvation (Expt. 4, Table III, and Expt. 7, Table VI). It is therefore evident that in none of these is the apparently

constant leaf number normally produced in continuous short days a true minimum, for treatments can be found which reduce it. Thus one may legitimately wonder whether any published 'minimum leaf number' is authentic or whether not all the factors governing the relationship between leaf number and flowering have been determined. Thus by comparison with Cooper and Money-Kyrle's *Lolium* experiments (1952), one may wonder whether the leaf number even of Petkus rye might not be reduced below 7, say by applying long-days (or better still continuous light) and vernalization temperatures concurrently. Gott, Gregory, and Purvis (1955) have recently, indeed, found such to be the case and have had, perforce, to suggest a lower number (5.6) as the true minimum.

They do not, however, reject the idea of minimum leaf number itself, because they found a convergence of the lowest leaf numbers produced by two quite different treatments: continuous light with concurrent vernalization as just mentioned; and the use of immature grain to reduce the number of primordia already present at germination. It might be remarked that it was just such a convergence—in that case between the leaf numbers for vernalized winter rye and unvernallized spring rye—that apparently influenced Gregory's original acceptance of 7 as the minimum for this plant. It would be well then to examine critically some of the other proposed vegetative phase determinants as possible alternatives to 'minimum leaf number'.

Anatomical maturity (A a 2) was tested for *Eupatorium* in Expt. 4 by determining 'linear densities' after the manner of Dormer (1951). However, it is obvious that the range of weights for equivalent internodes (Table III) is even wider than that of leaf numbers in the same experiment and there is thus no evidence that the onset of flowering is associated with any particular degree of thickening of the stem. As for (A b 1) and (A b 2) the small woody plants produced under conditions of mineral starvation flowered later than those fully manured in spite of the smaller number of leaves produced. This was especially true of *Eupatorium* as mentioned on p. 399 (Expt. 4) and *Celosia* in Expt. 7. Size (A b 3) likewise, whether measured by dry weight, leaf area, or height (Tables III and VI), appeared quite independent of the onset of flowering. Indeed, it may be said that of all the measures of the vegetative phase suggested, it is leaf number which shows the closest approximation to constancy and which therefore may be accepted as the best measure of physiological time so far available. Even if, then, one may doubt that it really indicates the attainment of ripeness-to-flower, some explanation of its constancy is called for: e.g. one or other of the suggestions in (B) and (C). In (B b) reference is made to some experiments of Barber and Paton (1952) concerning leaf numbers in pea varieties which, being genetically controlled, strongly suggest true minimum leaf numbers. Barber and Paton, however, found that the leaf number characteristic of a particular variety was reduced if the cotyledons were excised in the early stages of germination. They therefore suggest that a flower inhibitor is produced in the cotyledons and look upon leaf number merely as a measure of the time taken before the inhibitor is exhausted. Now

in Expt. 3 a cotyledon-removal treatment was applied to *Xanthium*, the intention there being to test whether there may not be a considerable carry over of mineral nutrients in the cotyledons and thus perhaps to impose a severer mineral deficiency in the early stages of growth. The treatment, indeed, did have a severe effect on general growth rate and also the time to flowering was increased even though, as in the pea experiments, leaf number was reduced (Table II). There was thus no evidence of inhibition of flowering by the cotyledons; on the contrary, both flowering and vegetative growth were inhibited when the cotyledons were removed, and the lowered leaf number is plausibly explained as a severer effect on vegetative growth. On the other hand, by considering, as Barber and Paton do, that leaf production is a measure of physiological time, it becomes possible to account for the usual leaf number of *Xanthium* without recourse to any concept of a stage of ripeness-to-flower.

Dissections showed that one pair of leaves in addition to the cotyledons is already present in the ungerminated seed of *Xanthium* and that two more leaves can usually be found a single day after soaking, thus it is necessary only to show that at the most 4 more leaves are initiated during the time required for reaction to short days which can hardly begin before the cotyledons at least are expanded. In a small experiment not described above, it was indeed shown that an average of 2.6 leaves may be produced after transfer to short days by plants that have already attained more than 9 leaves in long days. Roughly constant leaf numbers when *Xanthium* is grown in short days from germination would then be expected if it can be assumed that factors which affect rate of leaf production affect rate of reaction to daylength almost equally.

For *Eupatorium*, however, an equivalent explanation of the observed leaf numbers is not adequate. In Expt. 4, where the effects of mineral deficiency were tested, the reduction of leaf number was surprisingly small in spite of a 12-fold average difference of size (dry weight) between the sand and soil cultures (Table III). The seeds of *Eupatorium* are small and it was necessary to cut microtome sections to estimate the number of leaf primordia present in newly germinated seedlings, and even a week after germination not more than two pairs of leaves additional to the cotyledons could be found. In Expt. 6 the increase in leaf number during short-day induction was estimated by comparison of the final leaf numbers with the number present on the day short-day treatment was begun, derived from dissections of similar plants. The estimated increase varied from 7 to 12, with an average value of 9.5. Although this is a larger number than was found for *Xanthium*, it is by no means enough to account for the much higher leaf numbers produced in continuous short-day treatment, viz.: some 20 leaves are still unaccounted for by either the leaves present in the seedling or by those produced during induction. It will be noticed (Table V) that the leaf numbers found in Expt. 6 are not much greater than those of Expt. 4, even though in the former short-day treatment was not begun until nearly 5 months after germination. Thus in *Eupatorium* there does seem to be a period of vegetative growth before the plant becomes

ripe-to-flower, for it appears that all the short days received between germination and the attainment of some 20 leaves must have been ineffective in Expt. 4. A similar relationship between the effectiveness of induction and plant age was found by Borthwick and Parker (1938 and also 1940) for soybean. It is then necessary to assume that ripeness-to-flower here corresponds with a stage of development which is very closely correlated with leaf number. This is demonstrated with greater force by the experiments with cuttings. Thus in Expt. 5 the number of leaves produced by each cutting was independent of the number of leaves the stock plant had produced when the cutting was taken (Table IV). Moreover these numbers (average 26.8) agree almost exactly with those of the greenhouse-stock cuttings of Expt. 4 (Table III) which were derived from a much older plant than any represented in the series. They agree too with a small experiment not described above, in which cuttings all from one plant raised in long days were trimmed to have initial leaf numbers ranging from 4 to 12. There, independently of the original size of the cutting, the final leaf number on transfer to short days fell within the range 20–25 with an average of 23.1.

Doubt must, of course, always remain in such a case as this whether there may not be factors in addition to daylength which would further hasten flowering and reduce leaf number. It has been mentioned that this plant's behaviour in the tropics raises the suspicion that it is vernalizable, and though one must suppose that in all the experiments in Otago vernalization occurred naturally, it is easy to believe that the combination of temperatures and daylength may not have been the optimum possible. The revisions which Gregory and Purvis have had to make of rye's minimum leaf number on discovering progressively superior vernalization treatments provide sufficient precedent for this caution.

In *Celosia* too we are confronted with a relatively high leaf number, but here the reduction in leaf number produced by starvation was also high (Table VI) compared with what was effected in either *Xanthium* or *Eupatorium*. So, although the number of leaf initials present in the seed cannot, judging by the condition on germination, make a large contribution to the final leaf number, it would be unnecessary to invoke a minimum leaf number for this plant if as few as 3 further leaves can be accounted to initiation during daylength induction.

NOTE ON THE PROCESS OF INITIATION IN *EUPATORIUM*

As the flowering behaviour of *Eupatorium* had not previously been reported, it was necessary as described above to perform some preliminary tests before this plant could be used in leaf number experiments. In Expt. 6 some further information on initiation was obtained from the series returned to long days after brief exposure to short days and also from the sections of apices. A minimum exposure of some 24 days was there found necessary to complete short-day induction. One 24-day and one 16-day plant showed (by loss of

apical dominance) (Fig. 2) evidence of partial induction but otherwise it appeared that given sufficient short-day treatment to complete initiation the remaining stages of flowering followed spontaneously: every plant which produced an inflorescence also proved capable of setting seed even though its development was somewhat delayed in long days.

Since only duplicate microscopical preparations were made of each stage of induction, and, at least in the later stages, progress was variable, it is not easy to be certain at what morphological stage induction became irreversible.



FIG. 2. *Eupatorium adenophorum*, Experiment 6. From left to right: continuous short days; induction with 32 short days, 24 short days, 16 short days, and 8 short days. Condition shown on 8 December, induction having in each case begun 1 August.

It will be seen (Fig. 1a) that in wholly vegetative plants the apex itself is concave (this is confirmed in the T.S.s by the appearance of a hole in the ultimate section of the tip). The first apparent sign of a short-day effect is the widening of this depression, presumably because the immediate daylength effect is to inhibit the production of lateral appendages. Subsequently the apex becomes flattened and ultimately convex, presumably for the same reason. At about the same time (between 21 and 28 days) the T.S.s show the origin of a primordium out of alignment with the decussate phyllotaxis. This is presumably the first bract of the inflorescence, for phyllotaxy in the inflorescence does change to spiral. Subsequently, both L.S. and T.S.s show the elaboration of the inflorescence primordia, additional bract and bud primordia obviously differentiating very rapidly between the 28th and 35th days. So far as these observations then are reliable, it would appear that induction becomes irreversible at a definite morphological stage, namely,

when, following a cessation, primordium production is renewed at the apex with the initiation of the inflorescence itself.

ACKNOWLEDGEMENT

The greenhouse described was equipped with the aid of a grant from the Research Fund of the University of New Zealand.

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Study of Phenolic Compounds in Oil-Flax (*Linum usitatissimum*)

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Received 7 March 1956

SUMMARY

The complexity of the phenolic constitution of flax tissues is demonstrated. A method for the purification of phenolic compounds and their estimation from a complex mixture of these substances in plant extracts is described together with the use of semi-micro paper chromatography and paper electrophoresis for their identification.

Chlorogenic acid and an isomer of this acid are shown to be present in four linseed varieties. The concentration of chlorogenic acid and the ratio of chlorogenic acid to total phenolics is studied in relation to disease resistance.

INTRODUCTION

PHENOLIC compounds have long been considered to play an important role in the biochemical aspects of host-parasite relationships. Protocatechuic acid and catechol were demonstrated in the scales of onion varieties resistant to smudge (*Colletotrichum circinans* (Berk.) Vog.) but not in susceptible varieties (Angell, *et al.*, 1930). Kargopolova (1936) showed that the cell sap of wheat varieties highly resistant to leaf-rust (*Puccinia triticina* Eriks.) was characterized by a high concentration of phenolics of the protocatechuic acid type while the sap of susceptible varieties was poor or entirely devoid of these substances.

More recently Johnson and Schaal (1952) demonstrated the presence of chlorogenic acid in high concentration in the region of the periderm in all potato varieties resistant to scab (*Actinomyces scabies* (Thaxt.) Güssow) and that the chlorogenic-acid concentration varied with the degree of resistance of the variety. Harris (1953) reported that certain derivatives of the phenolic fraction of apple and pear sap markedly stimulated or inhibited growth *in vitro* of the scab fungus (*Venturia* spp). Kirkham (1954) has demonstrated by leaf-injection methods the toxicity of chlorogenic acid and isochlorogenic acid *in vivo* towards the two *Venturia* species.

In view of the possible importance of phenolic compounds in the study of the relationship between *Linum*-rust (*Melampsora lini* (Ehrenb.) Lev.) and flax varieties (*Linum usitatissimum* L.) a study was made of these compounds

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in healthy linseed tissues with special emphasis on chlorogenic acid. Methods available for the estimation of chlorogenic acid (Johnson and Schaal, 1952) were found to be reliable only for simple phenolic mixtures. A modification of these methods was necessary to study the complex group of phenolics present in linseed tissue.

In this paper is reported the occurrence, identification, and estimation of chlorogenic acid from a complex of phenolic compounds by the use of paper chromatography, paper electrophoresis, and ultraviolet spectrophotometry.

MATERIALS AND METHODS

Four linseed varieties, Williston Golden (C.I.¹ No. 25-1), Ottawa 770B (C.I. No. 355), Argentine Seln 462 (C.I. No. 462), and Bombay (C.I. No. 42) were grown in an unheated glasshouse in flat boxes (15 in. \times 12 in. \times 3 in.). Four box-replications of each variety were sown, samples of leaf and stem tissue from plants 12-15 in. high were taken for chemical analysis. Samples were cut from each box at 8.0-8.30 a.m. on the same day and replicates of each variety were bulked and the results based on one analysis.

Extraction. The leaf and stem tissues (50 g. fresh wt.) were extracted by dropping into 200 ml. 80 per cent. ethanol at room temperature and macerating in a Waring blender for 3 minutes. The extract was filtered on a Buchner funnel and the residue washed with 50 ml. 80 per cent. ethanol and re-extracted with 200 ml. 80 per cent. ethanol at 70° C. for 3 minutes, filtered, the filter cake washed with ethanol, and finally sucked dry.

The alcohol was removed by evaporation in a rotary film evaporator (Partridge, 1951), the aqueous extract was clarified by filtering through Hyflo-super-cell (30 gm.). The phenolics were removed by absorption on carbon and desorption with hot ethanol (Nordström and Swain, 1956). The final alcoholic extract was reduced to a standard volume (5 ml.).

Purification. Two-dimensional chromatograms (46 \times 55 cm.) were run using water-washed Whatman's No. 3 (thick) filter paper and the following solvents: 1st: *n*-BuOH:HAc:H₂O (6:1:2 v/v) (Nordström and Swain, 1953). 2nd: acetic acid (6 per cent.) (White, Kirby and Knowles, 1952).

The phenolic extract was applied from a self-filling micro pipette in 5 μ l. aliquots to the corner of a large Whatman No. 3 filter paper. The spots obtained were well separated and no trailing was visible when viewed under ultra-violet light (Fig. 1). The major spots were marked under ultra-violet light, cut out, and chromatographically eluted with an aqueous ethanol solution (50-70 per cent.). By preparation of several chromatograms as described above relatively large chromatographically pure samples of the major components were obtained from the crude phenolic extract.

Identification of phenolic compounds. In no instance were the compounds to be identified available in more than microgram quantities. The following types of evidence were obtained. (a) Chromatographic. Chromatographically

¹ C.I. No. refers to accession number of the Division of Cereal Crops and Diseases, Department of Agriculture, United States of America.

homogenous solutions were analysed by their behaviour in several different solvent systems on semi-micro ascending strip chromatograms (Redfield, 1953) under standard conditions. A standard synthetic compound (phloroglucinol) was run on all papers (Bate-Smith and Westall, 1950). (b) Chromogenic. The appearance in visible light and under ultra-violet light with and without ammonia vapour, and the colours given by several chromogenic spray reagents, were recorded (Swain, 1952). (c) Spectrographic. The ultra-violet absorption spectrum was determined in distilled water or aqueous ethanol

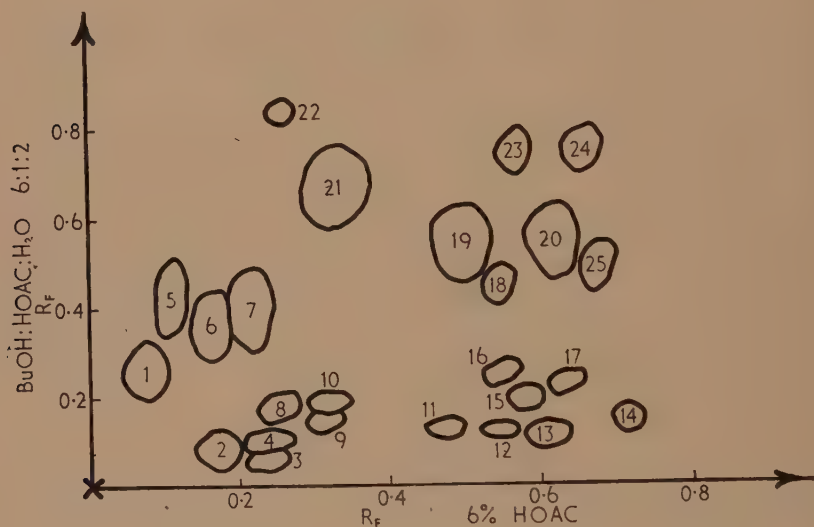


FIG. 1. Chromatogram of phenolic compounds in linseed extract.

(2.5 ml.) with and without the addition of 0.5 ml. of 0.002 M. sodium ethoxide (Mansfield, Swain, and Nordström, 1953). (d) Paper electrophoresis. The electrophoretic mobilities in terms of R. caffeic values were determined using an 'EEL' paper-strip electrophoresis apparatus. The papers were run at 450 v. and 20 ma. for 2 hours using a borate buffer solution of pH 9.2. The strips were dried and viewed under ultra-violet light to determine the position of the spots. (e) Hydrolysis. The samples were subjected to acid hydrolysis (100° C. for 1 hour with 2N/ H_2SO_4) and the hydrolysis products determined (Nordström and Swain, 1953).

Estimation of chlorogenic acid. Chlorogenic acid was separated from 5.9 μl . aliquots of the crude phenolic extracts by ascending two-dimensional chromatography on water-washed Whatman's No. 3 (thick) filter paper (28 \times 28 cm.) using the solvent pair described above.

The spots corresponding to the known position of chlorogenic acid were marked under ultra-violet light, cut out of the chromatograms, and eluted chromatographically with copper-free distilled water directly into 5 ml

volumetric flasks. Spots of similar area from blank sections of each chromatogram were also cut out and eluted. Approximately 2.5 ml. were collected during elution and the eluate made up to standard volume with water. The extinction coefficients of the solutions were measured against their paper blank solutions in a Hilger 'Uvi-Spec' spectrophotometer at 324 m μ . The latter were converted to mg./g. fresh weight of plant tissue by reference to a standard calibration curve. Triplicate determinations were made.

Estimation of total phenolics. The λ_{\max} in ethanol of the crude phenolic extract was determined by plotting its ultra-violet absorption spectrum. The extinction coefficients of the four extracts to be compared were measured at this wave-length (330 m μ). No absolute determination was made, but the extinction coefficients were constant and could be used as a measure of the total phenolics present for comparative purposes.

RESULTS

The characteristic pattern of spots due to the presence of the phenolic compounds in linseed tissues obtained on two-dimensional chromatograms is shown in Fig. 1.

These compounds fall into three distinct groups. Firstly those (spots 1-10) which give a dark-brown fluorescence in ultra-violet light. This group appears flavone-like (similar to apigenin) on the basis of their chromatographic (Cruickshank, unpublished), chromogenic, and spectrographic characteristics.

TABLE

Identification of chlorogenic acids from linseed

	Eluate from Spot 19	Eluate from Spot 20	Chlorogenic acid from coffee
RF <i>n</i> . BuOH:HOAc:H ₂ O, 6:1:2 . . .	0.53	0.53	0.53
RF 6% HOAc	0.55	0.61	0.55
RF Phenol:water	0.47	0.47	0.47
RF <i>n</i> -BuOH:water	0.14	0.14	0.14
R _{caffeic} electrophoresis	0.65	0.65	0.65
U.V. λ max EtOH-aq	324	324	324
λ max NaOEt	380	375	380

Colour reactions were identical for all three materials: U.V. fluorescence, blue, going green with NH₃ vapour; AgNO₃+NH₃, brown; Alcoholic FeCl₃, bluish-grey: U.V. fluorescence with alcoholic aluminium chloride blue going yellow green with NH₃ vapour: Hydrolysis products were shown to be caffeic and quinic acids in all three cases.

Chlorogenic acid content of linseed

Variety	Chlorogenic acid mg/g. Fresh wt.	Ratio	$\frac{\text{Ext. coeff. Chlorogenic acid}}{\text{Ext. coeff. Total phenolics}}$
Williston Golden	4.41		0.267
Ottawa 770B	2.65		0.451
Argentine seln. 462	3.10		0.364
Bombay	3.27		0.400

On acid hydrolysis, however, no typical aglycone could be obtained. The compounds represented by spots 19 and 20 form a second natural group. These were identified as chlorogenic acid and an isomer of this acid (Table 1, fig. 2).

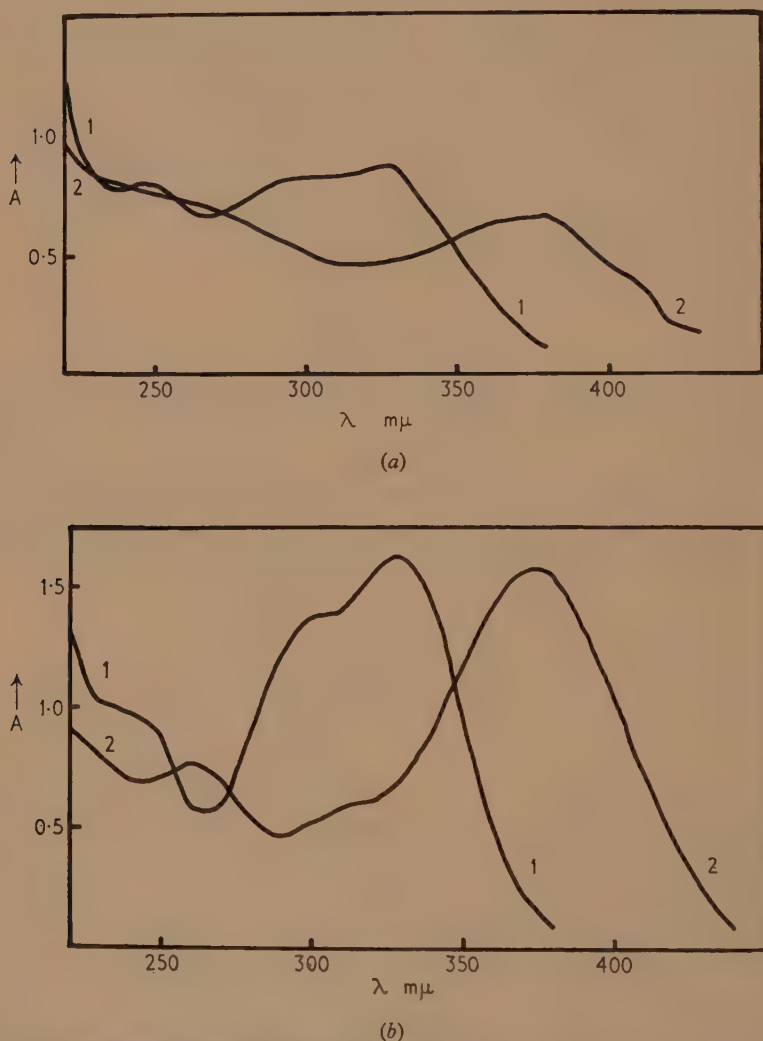


FIG. 2. Absorption spectra of eluate from (a) spot 20, (b) spot 19, in (1) EtOH-aq, (2) NaOEt solution.

A number of minor compounds constitute the third miscellaneous group. Most of these compounds are blue, green, or violet fluorescing and many show more intense fluorescence in the presence of ammonia.

The results of chlorogenic acid estimations in four linseed varieties and the ratio of the extinction coefficients of chlorogenic acid to that of total phenolics of the same variety are given in the Table.

DISCUSSION

Chlorogenic acid and an isomer of this acid have been identified in four varieties of linseed. In relation to their resistance to physiologic races of *M. lini* as a group, the variety Williston Golden is susceptible and the other three are resistant (Cruickshank, 1952). Williston Golden has a high concentration of this acid, the rust resistant group on the other hand have low concentrations. Determinations of the ratio of chlorogenic acid to total phenolics (Table I) showed that this ratio was highest in the highly resistant variety Ottawa 770B, and least in the susceptible variety Williston Golden.

The authors wish to thank Dr. E. C. Bate-Smith, Low Temperature Research Station, and Dr. S. Dickinson, School of Agriculture, for their continued interest in these studies. As far as one of us (I. A. M. C.) is concerned the work described in this paper was carried out during the tenure of a Nuffield Travelling Fellowship (Natural Sciences). As far as the other (T. S.) is concerned the work was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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Observations with the Electron Microscope on the Internal Structure of the Spermatozoid of *Fucus*

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Received 3 December 1955

SUMMARY

After preliminary description of antheridial dehiscence, details have been given of the internal structure and position inside the body of the *Fucus* spermatozoid of the following organs: mitochondria, chromatophore and eyespot, ciliary bases, proboscis. The position and approximate shape of the nucleus both expanded and in the coiled condition are described. The body membrane and its relation to the covering membranes of the proboscis and two flagella are described. The plane of symmetry of both flagella relative to the surface of the body is described.

The more important individual facts recorded are:

1. The plane of symmetry in both flagella is at right angles to the body surface, passing between but not through the two central strands in each flagellum.
2. A group of dark bodies previously mistaken for the nucleus are mitochondria.
3. The proboscis is part of the ciliary apparatus; it is covered on each side by a membrane and the internal thickening bands are attached at one end to the basal body of the front flagellum.

INTRODUCTION

THIS work began as a study in the bilateral symmetry of cilia, its object being to correlate their internal structure with the orientation of their external appendages, by means of thin sections. It is scarcely possible, however, to study any one organ in isolation from the rest of the cell and in this particular case the cell presents so many unusual features that to elucidate it even in outline has required a major investigation.

The difficulty of interpreting the microanatomy of the *Fucus* male cell has been felt by 'light' microscopists for nearly a century. With the new information now at our disposal we can see this to have been due in part to the exceptionally labile nature of some of the cell components, in part to their unusual shapes, and in part to a chemical peculiarity possessed by a group of large mitochondria which under certain conditions may cause them to show a misleading resemblance to the nucleus of more ordinary cells. This has led to a sharp difference of opinion regarding the position and size of the nucleus which has been much discussed (cf. Fritsch, 1945) but which is still not resolved (cf. our own previous account, Manton and Clarke, 1951).

In the work which follows we have concentrated our attention on the microanatomy of the cell as a whole, using a variety of methods. Most of the appearances which have been encountered in various ways by light micro-

scopists have confronted us and have had to be explained. In doing so we have presented enough details in the plates to authenticate the identity of the various organs and to give some of the more obvious facts about their structure. We have not attempted to do more than this. Many details could be further pursued with profit and some reference back to our previous work (Manton and Clarke, 1951 and Manton, Clarke, and Greenwood, 1953) will be necessary to supplement the present account with the facts of external morphology which are already known.

MATERIAL AND METHODS

There is nothing new about the source of material. Male plants from the Yorkshire coast, stored in a refrigerator overnight, were rinsed in chilled filtered sea-water and the exudate left near a window to dehisce. The motile spermatozooids accumulate on the side away from the window if left undisturbed for half an hour.

Before ultraviolet photography and for direct preparations for electron microscopy, killing was by 30 sec. exposure to the vapour from a 2 per cent. osmium tetroxide solution. For ultraviolet microscopy the material was killed on a quartz slide and immediately covered with a quartz coverslip to form a sealed liquid mount, the growth of bacteria having been suppressed by the addition of a drop of iodine solution to the sea-water. For direct preparations for the electron microscope, the material, after killing, was merely dried, washed, and shadow-cast with gold-palladium.

Fixation for embedding was for 1 hour in 2 per cent. osmium tetroxide solution diluted with an equal volume of acetate-veronal buffer (pH 8.2 and 7) all made up in distilled water. The living material was either drawn up in a pipette from the light concentrate and squirted into the fixative, thereby diluting it further by an uncertain amount, or the light concentrate was spun down in a centrifuge and the fixative poured on. Both methods gave usable results, but the first, in spite of its uncertainty, is to be preferred. To test fixation, some direct preparations were made from the fixed material before further processing and several of these are shown.

Embedding was into a mixture of 9:1 butyl: methyl methacrylate solidified with the commercial catalyst Luperco in an oven at 46° C. Before embedding, the fixative was rapidly rinsed out with two changes of buffer and the material dehydrated through the usual mixture of alcohols, using a centrifuge between each, at room temperature.

Sections were cut on a Porter-Blum type 'Servall' microtome (cf. Porter and Blum, 1953) in the Leeds Botany Department, using a glass knife.

Observations were carried out in several different laboratories. The ultraviolet microscopy was done in Leeds using the Cooke, Troughton, and Simms microscope in the Botany Department. The low-power electron micrographs were done on the Philips electron microscope in the Leeds Botany Department supplemented by a similar machine in the research laboratories of Messrs. Tootal Broadhurst Lee Ltd. of Manchester. Micrographs taken on

the latter instrument are: Figs. 2 and 3, Pl. I, Fig. 6, Pl. II, Fig. 7, Pl. III, and Figs. 11 and 12, Pl. IV. Lastly, all the high-power observations contained on Pls. VI to XI were made on the RCA microscope in the Department of Anatomy of Harvard Medical School, Boston, U.S.A.

Note on fixation artefacts. It is not always easy to detect artefacts, still less to avoid them. For this reason it is desirable to draw attention to some of the more troublesome of those which we have encountered since they can give rise to serious difficulty in understanding preparations. We have been troubled by three main types of defect:

1. All our early sections after alkaline fixation (pH 8.2) showed apparently good fixation of the nucleus (once this had been finally identified) but manifestly bad fixation of other cell components especially those involving membranes. Examples are reproduced in Figs. 13–15, Pl. V, and in Figs. 18–19, Pl. VI. Loss of membranes was much less prevalent with neutral fixation and all our best sections, notably those on Pls. VIII and XI, were fixed at pH 7.

2. Loss of opacity by solution of electron opaque components, with or without loss of membranes, can affect several organs. The pigments of the eyespot can most easily be traced since they colour the high alcohol concentrations (70 per cent. to absolute) in which they are soluble. They dissolved out completely from our material fixed at pH 8.2, though it is not quite certain whether this was primarily due to the alkaline fixation or to slow dehydration which was carried out at the same time in the hope of hardening some other cell components. The eyespot pigments were retained best with rapid dehydration after neutral fixation in material which had been centrifuged in the living state without subsequent breakage of the clot until the methacrylate was reached. Which of these various things was most decisive for retention of the pigments is uncertain.

Loss of opacity by the nucleus occurs in two ways: it may lose its electron-stopping power without apparent loss of structure or the entire centre of the nucleus may appear empty and hollow. We have not illustrated the first condition but the second appears incidentally in Figs. 32–35, Pl. X. Both may have been concerned in the production of the very misleading condition of 'transparent bodies' which we encountered previously as a rare appearance after vapour fixation. The immediate cause of either is unknown and the state of the material before fixation may perhaps have something to do with it. Affected blocks must be discarded, since both these conditions add very greatly to the difficulty of interpreting preparations.

Loss of opacity in the mitochondria, which occurs at a different rate from that of the nucleus, is more difficult to demonstrate since it is usually so complete that in sections of all our blocks the mitochondria have become almost completely transparent except for their external and internal membranes. That they are probably not so in life is suggested by appearances such as those of Figs. 9 and 11, Pl. IV, which have been of critical importance for sorting out the controversy regarding the position of the nucleus (pp. 422–3 below).

3. Blistering, due probably to osmotic activity occurring beneath intact semipermeable membranes immediately after death of the cell in the early stages of fixation, also affects several organs. We have long been familiar with this with respect to cilia and beyond noting that there are several examples included in the field of Fig. 2, Pl. I, we need not discuss it. That blistering can also occur from the edge of the proboscis (Figs. 2 and 3, Pl. I) is a new observation which could not be interpreted until the compound nature of the proboscis had been elucidated (see below, p. 425). A third example is inside the mitochondria. Even our most perfect sections in otherwise well-fixed material are disfigured by what look like large holes torn within the mitochondria (cf. Figs. 26 and 28, Pl. VIII). Whether these are osmotic effects of post-mortem changes inside the mitochondria, or are connected with our method of changing the salt concentration of the surrounding medium in transferring from sea-water to the various aqueous solutions, is unknown. A preliminary experiment of replacing distilled water in the fixing solution by sea-water met with no success at all. This particular artefact has therefore at this stage had to be tolerated.

OBSERVATIONS

Dehiscence of the antheridia. Pl. II, Fig. 5, shows the appearance of newly emerged spermatozooids as seen with the ultraviolet microscope. The position of an empty antheridial membrane is indicated by an arrow and spermatozooids in various states of uncoiling are scattered in the sea-water. A more highly magnified view of a coiled and an uncoiled cell (still in sea-water) is given in Fig. 4. It is important to notice the change of shape from the spherical condition to the elongated condition which is associated with uncoiling since both these states will be encountered in sections. The information on position of the two cilia, of the proboscis and of the eyespot which are discernible in the lower cell of Fig. 4, is also important for correlation with the sections.

Fig. 6, Pl. II, gives a low-power electron microscope view of a dried empty antheridial membrane which can usefully be compared with that of Fig. 5 and with the sections of Figs. 7 and 8. The membrane is characteristically open at both ends in this species as may be seen in Figs. 5 and 6, but when cut obliquely, as is usually the case in random sections, only one open end may be exposed (Figs. 7 and 8), but this is not a true picture of a median view.

Comparison of the two sections reproduced on Pl. III gives a preliminary insight into the problems involved in interpreting the cell structure. Fig. 7 shows good fixation (pH 7) in which cell membranes and cell inclusions of various kinds, including the large and relatively opaque nuclei, are well preserved, but all the cells present in the field are still in the coiled spherical state. In Fig. 8 an antheridium is almost empty and all the cells remaining in the field are uncoiled. Fixation (pH 8.2) is poor for membranes and the softer cell inclusions though the nuclei are still intact and display something of their bizarre appearance in various planes of section.

Morphology of the cell. It is essential to discuss the uncoiled condition of the cell first since it is too difficult to proceed in the reverse order. External views of intact dry cells are contained in Pl. I and these should be carefully compared with the undried cells of Pl. II, Figs. 4 and 5. The act of drying necessarily produces some distortion. There is some, though apparently not much, shrinkage and in certain attitudes there is lateral displacement of parts. In Fig. 1, for example, which is reproduced from our previous paper (loc. cit. 1951) the proboscis is in face view though the cell body is partly in side view. This figure nevertheless gives a very good display of the external parts of the cell including the garniture of hairs (*Flimmer*) on the front flagellum and the adhesion of the hind flagellum to the surface of the eyespot which are important features that will recur in our sections.

The material actually embedded is shown in Figs. 2 and 3. They add a few more details which were not visible after vapour killing (Fig. 1), notably the skin loosely covering the body surface (Fig. 2) and the configuration of the margin of the proboscis (Fig. 3) which will be further discussed below.

In all the figures of Pl. I the cell body is completely opaque to electrons and this is the usual condition of fixed material examined whole. Occasionally, however, the cell body may become partially transparent or even spread in a thin layer upon the formvar film, in which condition some of the more resistant internal parts may be revealed though in a state, as our experience shows, which may be very difficult to interpret correctly without the aid of sections. The interpretation which can now with confidence be offered for the three types of cell illustrated in Pl. IV thus hangs on a close correlation with sections of the same material but it will be convenient to examine these cells carefully at this point since they have contributed a great deal to understanding the cell as a whole.

Pl. IV, Fig. 9, reproduced from Manton and Clarke, 1951, shows a transparent cell in which the more conspicuous organs are labelled (*e* eyespot, *b* ciliary bases, *p* proboscis and connecting fibrils, and *m* the group of mitochondria). Most of these are self explanatory and were correctly interpreted in our previous paper. The dark mass *m*, however, which we now know to be composed of mitochondria was at that time mistakenly thought to be the nucleus. That it is not the nucleus is revealed both by the sections and also by the sum of evidence from the other types of transparent body reproduced in Figs. 10 and 11.

Fig. 10 is from the material fixed for embedding at pH 8.2. The cells are exceedingly soft after this treatment and the eyespot may even fall out intact (Fig. 12). In Fig. 10 the eyespot is still in position though partly dismembered, but the dark mitochondrial mass has vanished. Fig. 11, on the other hand, is after neutral fixation (pH 6.9). This compares very closely with Fig. 9 except that the cell is lying the other way round, but the dark mass behind the eyespot is now represented by three or probably four separate oval bodies. The identification of these bodies as mitochondria follows unequivocally from their internal structure as revealed by sections, and their disappearance from

material with the treatment of Fig. 10 can be correlated with the loss of internal membranes after alkaline treatment.

Since it is common experience that the nucleus is usually one of the more resistant cell organs and is normally relatively opaque, it is necessary to look elsewhere for it. The evidence of sections, e.g. Pl. V, gives the facts for relative opacity and, when these are projected on to the shapes remaining in the whole mounts, it becomes clear that most of the material hitherto referred to as 'body' is of nuclear origin. It is one of the most difficult things in the whole investigation to determine the exact shape of the nucleus in the various states of uncoiling in final detail since it is never possible to obtain a complete series of sections through any one cell. It is, however, not difficult to describe it in general terms. The nucleus is the largest organ in the cell body, filling it almost entirely and responsible for its shape. The chromatophore and mitochondria are partly accommodated in a hollow on one side and there is only a very thin layer of cytoplasm covering the other side. At the front end, which is attenuated into a beak-like extremity, there is a firm lateral attachment between the nucleus and the bases of the two cilia (Pl. VII). There is a very delicate bounding membrane covering the whole cell and continuing into the membranes covering the proboscis (Pl. XI) and cilia. There is very little space inside this membrane to accommodate any organs other than those listed.

In the coiled condition the nucleus is flexed sharply back on itself at a point immediately behind the attachment to the ciliary bases, giving the L.S. a somewhat S-shaped appearance, with the mitochondria and chromatophore accommodated in the lower loop of the S (Fig. 28, Pl. VIII, and Text-fig. 5).

All these facts are illustrated in the plates and some will be discussed further below. Attention may, however, perhaps usefully be drawn to the diagrams assembled on p. 428 which will be referred to from time to time in connexion with certain types of section and which summarize much of what has already been said on a basis of the outlines of some of the more critical figures which have already been introduced.

The eyespot. Since this is the easiest organ of the cell to recognize in all types of preparation, it is convenient to describe it first. Two complete specimens detached from their cells can be seen in Fig. 12, Pl. IV. The pigment facets, visible in these and in Fig. 10, Pl. IV, vary in number from 50 to 70. They occupy a circular or, more commonly, an oval patch in a characteristic place behind the ciliary bases. Their appearance in section can be seen in Pls. VIII and IX.

Fig. 30, Pl. IX, shows a tangential section of part of the eyespot at a level just below the surface. The facets, still retaining their pigment, are cut in cross section. When cut longitudinally as in Fig. 31 and elsewhere in Pls. VIII and IX they are found to be roughly cylindrical chambers each with a rounded base and flattened top and bounded by a wall which remains behind after loss of the pigment (compare Pl. VIII with Pl. VI). They are commonly about twice as deep as they are wide and it is evident that they are spread in a single layer on the outer side of the chromatophore.

The patch of pigment chambers is below the chromatophore membrane and is therefore definitely part of the chromatophore. It is also covered on the outer side by the body membrane (see Fig. 29, Pl. IX) to which the hind flagellum is attached by some kind of a cementing mechanism causing adhesion of a distended region of the flagellar membrane adjacent to the eyespot (Fig. 31, Pl. IX). The rest of the chromatophore contains a few septa somewhat buckled and irregularly arranged and between these in life there was probably some osmotically active material which has dissolved out before embedding since the distended spherical shape of the whole organ, at least in unexpanded cells (Figs. 27, 28, Pl. VIII), suggests that they were turgid before fixation.

The mitochondria. The internal structure of mitochondria is now sufficiently familiar to electron microscopists to be recognized at sight as soon as satisfactory sections are available. This has already been done for the *Fucus* egg by Wettstein (1954) and in the spermatozoid they are very similar. Fig. 24, Pl. VII, shows two large mitochondria partly accommodated in a depression in the nucleus in the region behind the eyespot. Less highly magnified sections showing the relation of the mitochondria to other organs of the cell will be found on Pls. VI, VIII, and IX. The characteristic feature diagnostic of mitochondria is the presence of internal invaginations of the inner membrane. These invaginations, or 'cristae' as they have been named by Palade (1952, 1953), are finger-like in *Fucus* as they also are in the Flagellate *Synura* (Manton, 1955), whereas in many animals they are plate-like. In all cases, however, the cristae project into the lumen of the mitochondrion without entirely filling it. They are, however, relatively numerous in this material and the large cavities apparently torn in the mitochondria in Figs. 26 and 28, Pl. VIII, are fixation artefacts as already explained.

The only other structural detail revealed by our sections is the suggestion of alignment of the cristae at right angles to the surface where the end of a mitochondrion is pressed against the chromatophore (Fig. 31, Pl. IX). This could have a functional significance though the evidence is still too slight to discuss it further.

With regard to the number of mitochondria present, it is usual to encounter 1, 2, or 3 in a cross section of the cell though random sections are an insufficient guide to the total complement. The work of some of the older light microscopists is of special value here since although the mitochondria have not previously been identified as such they have been seen and closely studied several times. Outstandingly good papers are those of Retzius, 1905, and Meves, 1918. The former gives the number as predominantly 4 but occasionally 5, the latter describes one large mass, or alternatively 3, 4, or 5 smaller ones. We may discount some of Meves's alternatives as referring to a compacted group such as that of our own Fig. 9. The main weight of evidence thus points unmistakably to 4 and with this our own Fig. 11 is in agreement.

We have already commented upon the loss of opacity of the mitochondria during embedding which is attested by the comparison of the same material

dried (Pl. IV and p. 418). We have no knowledge of the nature of the opaque component which has been lost but it is likely to have been osmotically active since the mitochondria, like the chromatophore, suggest by their shape that they must have been turgid in life and sufficiently rigid to cause depressions in the nucleus of the coiled spermatozoid (Fig. 26, Pl. VIII).

The cilia. Transverse sections of the two flagella have been included incidentally in many of the sections already referred to on Pls. VI to IX. Fig. 29, Pl. IX, is particularly good for demonstration of the general structure of the flagellar axis with its ring of nine strands round a central pair in the normal free part of the flagellum where it is merely covered by a close-fitting skin and without appendages. Longitudinal views are contained in Fig. 31, Pl. IX, Figs. 20 and 21, Pl. VII, the best median view of the ciliary axis being that of Fig. 31.

Our study of cilia in this material has been primarily concerned with two main topics, firstly the plane of symmetry and secondly the structure and spatial relations of the parts internal to the cell. It will be convenient to discuss symmetry first since one observation has already been published in a preliminary form (Manton, 1955a, 1956).

To determine the plane of symmetry in a cilium it is necessary first to be able to resolve separately the two central strands and then to determine their position relative to some external feature which in *Fucus* can be the lateral hairs on the front flagellum or adjacent parts of the body, notably the nucleus, proboscis, or eyespot.

For the front flagellum we have introduced three new specimens all confirming the facts for the one previously published (Manton, 1955-6). Fig. 24, Pl. VII, shows the front flagellum cut very near its base in alkaline-fixed material deficient in body membranes though the ciliary membrane is still present. The two central strands are clearly resolved in the original print if not in the reproduction, and the line joining them is roughly parallel to the surface of the flattened nucleus. A much better specimen not, however, related to a body is shown in Fig. 36, Pl. X. The two central strands are widely spaced and the position of one of the rows of lateral hairs is revealed by the fine lines on the left of the axis. The relation of these to the central strands shows that the plane of bilateral symmetry of the whole organ is vertical to the page and passes between but not through the central fibres. A still better specimen is contained in Figs. 37-38, Pl. X. These are two adjacent sections selected from a longer series which extended sufficiently far back into the cell to include the tip of the nucleus. This is just grazed in Fig. 37 as a narrow dark patch immediately behind the thin central region of the proboscis (compare with Text-fig. 1). In Fig. 37 the front flagellum is loosely invested by a dilated skin. This is more fragmentary in Fig. 38 but cannot be confused with the still more delicate lines of hairs marking the position of the lateral rows on each side. These show conclusively that the plane of symmetry passes between but not through the central strands of the ciliary axis, that it is perpendicular to the cell body and that the odd median fibril lies outwards relative

to the body. This is exactly the conclusion at which we had previously arrived (Manton, 1955a).

As in 1955, we are still uncertain about the exact mode of origin of the lateral hairs in relation to the fibrils of the peripheral ring. If the median fibril is numbered 0 and the others 1, 2, 3, 4 on either side, the origin of the Flimmer is almost certainly in close relation with fibril 2, but since fibrils 2 and 3 very commonly stick rather closely together, at least on one side of the axis in any one section, the possibility is open that the real place of origin of the hairs may be between these two fibrils. Comparison of Text-fig. 1 with Fig. 38, Pl. X, may perhaps usefully sum up these observations.



FIG. 1.

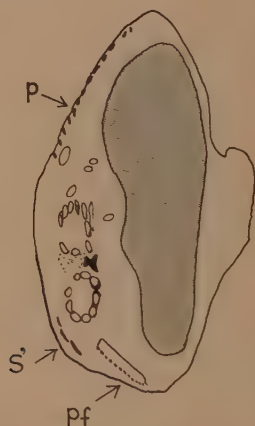


FIG. 2.

TEXT-FIG. 1. Drawing traced over the photograph of Fig. 37, Pl. X, to show the plane of symmetry of the internal strands of the front flagellum in relation to the lateral hairs, the tip of the nucleus, and the proboscis. For further explanation see p. 423.

TEXT-FIG. 2. Drawing traced over the photograph of Fig. 42, Pl. XI, to show the structure of the front end of the body at the level of the ciliary bases. Nucleus mechanically stippled, proboscis fibrils *p*, flange bearing proboscis fibrils on its front surface *pf*, tripartite strand connecting ciliary bases with proboscis surface *S'*. The basal body of the front flagellum is the lower one in this section. For further explanation see p. 426. Note the magnification of this figure is larger than that of Text-fig. 1 although orientation of the cell is similar.

The hind flagellum is in some ways more difficult to investigate since it possesses fewer external landmarks. The curious way in which it is stuck to the body membrane where it passes over the eyespot has already been noted (p. 422) and though the functional significance of this feature is entirely unknown the very marked dilation of the flagellar membrane in this region (see especially Fig. 31 and 29, Pl. IX) is certainly not an artefact since there is commonly well fixed protoplasm occupying the dilation. Within it the flagellar axis is eccentrically placed, being always nearer to the outer side to which it is perhaps organically attached. There is a curious band of diffuse dark material, marked with an arrow in Figs. 18 and 19, Pl. VI, and in Fig. 22, Pl. VII, which is perhaps involved in this. The band is not always visible in cross sections, perhaps owing to the distance down the eyespot at which the

sections have been cut, but when present it is very constant in position. It is always laterally placed, on the side of the flagellum towards the nucleus, and as nearly as we can determine it is in close relation to what we interpret as fibril 2 on the above terminology.

Whether this numerical diagnosis is correct or not there is no doubt that the two central strands of the flagellar axis run more or less parallel to the surface of the eyespot whenever they can be clearly resolved, which is by no means always the case. Figs. 18, 19, and 23 already quoted are some of our best examples. Where there is slight obliquity, which is not uncommon, we interpret this as an effect of accidental displacement since it is very inconstant in direction. This notwithstanding, the plane of bilateral symmetry is perpendicular, or almost perpendicular, to the body surface. The position of the odd median fibril, whether outwards or inwards, is slightly less certain. Most of our sections, e.g. Figs. 18 and 19, suggest that it is outwards, but the risk of misinterpreting this detail as a result of slight displacement of the fibrils is a real one (compare, for example, the two adjacent sections of one cell reproduced in Figs. 18 and 23). Our diagnosis of the orientation of the hind flagellum is therefore less certain than for the front flagellum though we have reason to think that both are in fact similar.

Contrary to what has previously been thought, at least for the hind flagellum, the only parts of the cilia which are internal to the cell are their basal bodies. These are shown in L.S. in Figs. 20 and 21, Pl. VII, and in T.S. in Fig. 25, Pl. VIII, and the two series reproduced in Pls. X and XI, which will be discussed in detail below. The basal bodies of the two flagella are attached together laterally, cf. Pl. XII, though they point in opposite directions (Fig. 14, Pl. V). They appear to be hollow chambers delimited from the free part of the flagellum by an opaque diaphragm which marks the level at which the body membrane passes over into the flagellar membrane (Fig. 31, Pl. IV). The central strands of the flagellum end in, or just below, this diaphragm. The wall of the chamber can be seen in T.S. (e.g. Fig. 42, Pl. XI, lower basal body) to be largely composed of the nine peripheral strands. Both basal bodies are in close contact with the nucleus, and the base of the front flagellum is organically attached to the nucleus by what appear to be fibrous connexions (Fig. 20, Pl. VII). Further discussion of the basal bodies will be found in the next section.

The proboscis. A great deal of new information about this mysterious organ comes from a few strategic sections. Its external appearance will already have been noted on Pl. I, and what we believe to be the normal swimming posture, with the proboscis directed forward, is seen in Fig. 13, Pl. V. After chemical killing the organ is often flexed sharply away from the body, as may be seen in Fig. 4, Pl. II, and this is the position in our best longitudinal sections reproduced in Figs. 40 and 41, Pl. XI. Fig. 40 is an L.S. of a proboscis near one side in which the section runs parallel to one of the bands of thickening (cf. Fig. 3, Pl. I). Fig. 41 is cut nearer to the centre; it grazes the base of the front flagellum and cuts obliquely across all thirteen of the thickening bands

of the proboscis. Both sections show unmistakably that these bands are internal and are enclosed on both surfaces by a membrane which is continuous with the body membrane. The capacity to form blisters as a post-mortem artefact at the edge of the organ, to which attention had already been directed (Fig. 3 and p. 419), is further confirmation of its compound nature.

The proboscis fibres start abruptly at the level of the ciliary bases as indicated in Fig. 40, Pl. XI, and further details can be made out from the two series of sections reproduced in Figs. 32–35, Pl. X, and Figs. 42–43, Pl. XI. When they first appear the fibres are borne on the external face (cf. Text-fig. 2) of a flat flange of material labelled *p.f.* in Figs. 33 and 42 which is joined at its lower end to the outside of the basal body of the front flagellum (Figs. 34 and 43). When the flagellum has left the body, the fibres remain within the body membrane as a row of parallel strands (lower *p* in Fig. 39, Pl. X) until the tip of the nucleus is reached when they arch forwards as in Fig. 3. On returning to the body on the other side of the arch they again form a row of parallel strands immediately inside the body membrane (upper *p* in Figs. 39, and 42) which passes near the base of the hind flagellum without touching it and then travels right down the body as may be seen in the transparent cell of Fig. 9, Pl. IV. This band can be readily picked up in sections at various levels, notably those in the region of the eyespot where it is constantly on the nuclear side of that organ (Figs. 17–19, Pl. VI) and even beyond it (Fig. 24, Pl. VII, right-hand side). At the back end of the body the strands appear to peter out gradually though the last two or three curve round the end and up the other side until they become lost to view over the opacity caused by the mitochondria (Figs. 9 and 11, Pl. IV).

Other parts of the ciliary apparatus. The proboscis is thus undoubtedly part of the ciliary apparatus and in fully dismembered cells the fibrous basis of the whole apparatus may come out intact (Figs. 45–46, Pl. XII). Such specimens also include one or both of two additional organs which were already noted in the transparent bodies described in Manton and Clarke, 1951, where they were designated as the strands *s* (loc. cit., Figs. 12–14). It will be convenient here to distinguish them as *s'* and *s* to denote fibrous connexions passing from the neighbourhood of the ciliary bases to the surface of the proboscis and the edge of the eyespot respectively.

The first of these strands (*s'* on Fig. 46) can be picked up readily in sections, e.g. Figs. 42, Pl. XI, and 39, Pl. X. It appears to be composed of at least three separate fibres which can be traced backwards to the region between the two ciliary bases and forwards up the front surface of one side of the proboscis for a distance equal to about five times the length of the ciliary bases when it ends in an unknown manner. For a better example of the distal termination of this organ on the proboscis face the reader is referred to Fig. 13 of our previous paper.

The other strand labelled *s* in Pl. XII is more difficult to identify in sections except when cut longitudinally (Fig. 44). It can then be seen to arise from between the two basal bodies and to pass backwards, as previously described,

towards the edge of the eyespot where it ends in an unknown manner. As may be seen from the dismembered specimens (Figs. 45 and 46, Pl. XII) this strand also is compound, at least two component fibrils being clearly separated in both figures.

Other parts of the cell. There is little further to say about other parts of the cell. That there is a body membrane surrounding everything is discernible in sections such as that of Fig. 16, Pl. V, though it is exceedingly delicate and correspondingly easily broken. Within it, apart from the nucleus, chromatophore, mitochondria, and ciliary apparatus, there is very little space for other structures although there is doubtless some. Unbroken sections such as those of Fig. 17, Pl. VI, Fig. 24, Pl. VII, Fig. 25, Pl. VIII, and Fig. 29, Pl. IX, show some additional vesicles and cavities which cannot, however, at present be chemically or morphologically diagnosed. Some of these spaces are probably due to shrinkage, as for instance in all the figures on Pl. X which show a wide apparent gap between the nucleus on the one hand and the body membrane and cilia on the other which cannot be a true picture. When allowance has been made for this there is very little unused space remaining, and there is equally little sign of either undifferentiated protoplasm or of unorganized food stores. The occasional fat bodies which turn up in sections, e.g. Fig. 18, left, are more commonly outside the cells than in them. They are almost certainly residual nutrients from a dehisced or broken antheridium since fat is so easily retained in these techniques that had it been a normal component of the swimming cells themselves it ought to be readily detected.

DISCUSSION

We are thus clearly dealing with an extremely specialized type of cell in which everything is subordinated and reduced to the barest minimum to permit the short life and limited function of a motile gamete. Even among gametes it is likely to be one of the most highly evolved types of cell to be found in the algae and it is perhaps unfortunate that the accident of accessibility should have caused us to study it first since otherwise a greater amount of interpretation in terms of comparative morphology would have been possible. Since it is obvious that we shall probably have to discuss it again on a comparative basis when more facts are available we have refrained on this occasion from pressing the interpretation of our sections to full finality, and we have not attempted to make a drawing purporting to reconstruct the cell since we are still in doubt about certain features of the shape of the most essential component, the nucleus. The extreme diversity in the plane of flattening shown by sections such as Fig. 17, Pl. VI, compared with Figs. 18 and 19 on the same plate, makes such a reconstruction extremely difficult and we are also unable to assess what degree of spiral twist there may be in the cell as a whole, though the rotation during swimming suggests strongly that there is some. We have therefore contented ourselves on this occasion with the diagrams assembled on p. 428, all of which are based on preparations included in the plates. In particular Text-fig. 4, based on Fig. 9, Pl. IV, has

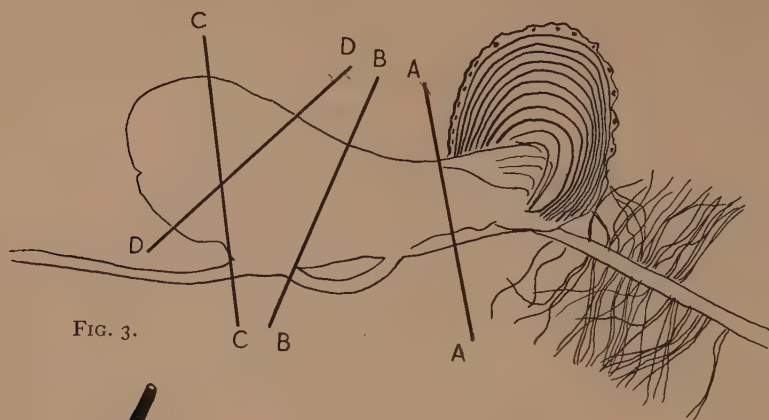


FIG. 3.

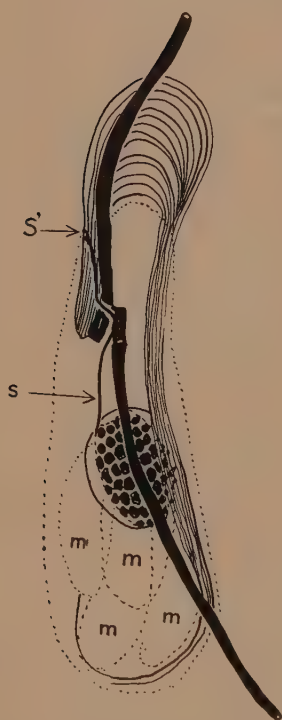


FIG. 4.

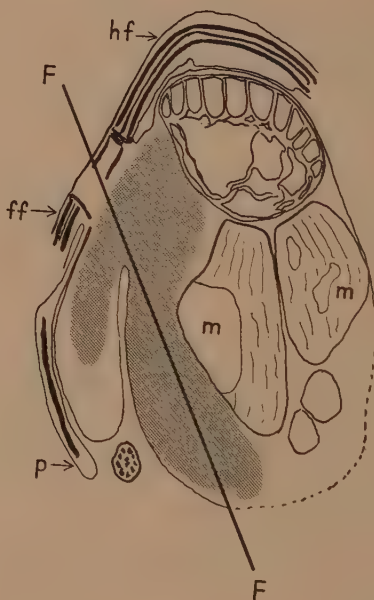


FIG. 5.

TEXT-FIG. 3. Diagram based on the outline of Fig. 1, Pl. I, with lines to indicate some of the principal planes of section. *AA* level passing through the ciliary bases as in Figs. 42-43, Pl. XI. *BB* and *CC* delimit the area through which sections regarded as transverse through the body in the region of the eyespot may have come, e.g. Pl. VI. *DD* section behind the eyespot in the region of the mitochondria (Fig. 24, Pl. VII).

TEXT-FIG. 4. Diagrammatic reconstruction of a face view of a dried cell based on Fig. 9, Pl. IV, for further explanation see text, p. 429.

TEXT-FIG. 5. Outline diagram traced over the photograph of Fig. 28, Pl. VIII, to show flexed position of the front end of the body in the coiled spermatozoid. *FF* the plane of section passing through the ciliary bases as in Fig. 25, Pl. VIII (very different in amount of nucleus traversed compared with *AA*), the nucleus mechanically stippled, *m* mitochondria, *p* proboscis, *ff* and *hf* front and hind flagella.

attempted to reconstruct the approximate outline of the cell before the flattening on to the formvar film had taken place. This is the nearest we can give at present to a diagram of the structure of the cell as a whole, but even this contains points of uncertainty. Thus although primarily based on the outlines of the organs in a dried specimen, we have attempted to reconstruct the approximate sizes of the mitochondria from sections by comparison with the size of the eyespot which appears to shrink less on drying; relative shrinkage of the other cell parts has not been allowed for. We have had to guess which way round the cell is lying, but comparison with the ultraviolet series of Fig. 4, Pl. II, suggests that we are seeing it with the eyespot surface upwards. Should we be wrong in this, however, the diagram may represent a mirror image of the true configuration. In either case the diagonal line of the cilia across the body probably correctly represents the position in life though naturally the flattening of all the organs on to one plane does not.

One of the more important of the facts which we have established is the very close physical propinquity between the nucleus, chromatophore, and mitochondria. This is very clearly seen in Fig. 17, Pl. VI, and in the various Figures on Pls. VIII and IX. It is one of the dominating features of the whole cell and it suggests strongly a metabolic interdependence of all these organs. It has already been suggested that the most probable site for a nutrient store is inside the chromatophore and the close association of mitochondria, nucleus, and parts of the ciliary apparatus with this organ further suggests that the ciliary movements are intimately connected with the metabolism of all these organs.

That the proboscis is morphologically part of the ciliary apparatus is one of the more surprising facts which have been discovered about it. It is also one which ought to make possible the detection of homologous structures in other cells which might perhaps give a clue as to its function. This work will therefore be continued.

ACKNOWLEDGEMENTS

Our very grateful thanks are due to the authorities of Messrs. Tootal Broadhurst Lee, Ltd., of Manchester for the use of their Philips microscope for one day a week for three months in the spring of 1955 and in particular to Dr. P. Chippindale for active co-operation. Similar thanks are due to the authorities of Harvard Medical School for the use of their RCA microscope during three weeks in July 1955, and in particular to Dr. Don Fawcett, through whom the arrangements were made, and to Dr. John Luft who stood by to give instruction and to service the machine at all hours of the day and night.

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DESCRIPTION OF PLATES

PLATE I

- FIG. 1. Spermatozoid of *Fucus serratus* after osmic vapour killing and formalin hardening (from Manton and Clarke, 1951). Magnification $\times 20,000$, reversed print.
- FIG. 2. Two cells after liquid osmic fixation (pH 6.9) before embedding, direct print. Electron micrograph M221.11, magnification $\times 5,000$.
- FIG. 3. Detail of a proboscis from the material of Fig. 2, showing the banding and new details of the edge. Electron micrograph M221.10, magnification $\times 18,000$.

PLATE II

- FIG. 4. Ultraviolet photograph of two spermatozooids of *Fucus serratus* shortly after liberation, the upper one still coiled, the lower one uncoiled. Glycerine immersion quartz objective, U.V. exposure 207.3a, magnification $\times 3,000$.
- FIG. 5. A field of newly emerged spermatozooids, some coiled and some uncoiled with an empty antheridial wall marked by an arrow. Dry quartz lens, U.V. exposure 206.5a, magnification $\times 600$.
- FIG. 6. An empty antheridial wall seen with the electron microscope. Electron micrograph M145.1, magnification $\times 3,000$.

PLATE III

- FIG. 7. Dehiscing antheridium (obliquely cut) and newly liberated coiled spermatozooids after neutral fixation (pH 6.9). Electron micrograph M222.4, magnification $\times 4,000$.
- FIG. 8. Dehiscing antheridium (obliquely cut) and uncoiled spermatozooids, after alkaline fixation (pH 8.2) with nuclei well preserved but body membranes badly preserved. Electron micrograph M182.4, magnification $\times 4,000$.

PLATE IV

- FIG. 9. A cell with transparent body after vapour killing, from Manton and Clarke, 1951, showing the outline of the flattened cell, the proboscis (*p*), the cilia and ciliary bases (*b*), the eyespot (*e*), and a dense body behind it (*m*) previously mistaken for the cell nucleus, magnification $\times 10,000$.
- FIG. 10. A transparent body after alkaline fixation (pH 8.2); for further explanation see text, p. 420. Electron micrograph M145.10, magnification $\times 10,000$.
- FIG. 11. A transparent body after neutral fixation (pH 6.8); letters as in Fig. 9. Electron micrograph M220.7, magnification $\times 7,500$.
- FIG. 12. Two isolated eyespots showing the pigment chambers, from the material of Fig. 10. Electron micrograph M237.16, reversed print, magnification $\times 15,000$.

PLATE V

- FIG. 13. L.S. of two cells to show nuclear shape and position of the proboscis in the fully uncoiled condition. Electron micrograph M184.1, magnification $\times 10,000$.
- FIG. 14. Oblique L.S. through a partly uncoiled cell to show the path of the two flagella in relation to the basal bodies and eyespot. Electron micrograph M190.30, magnification $\times 10,000$.
- FIG. 15. L.S. of an almost uncoiled spermatozoid to show the relation of the hind flagellum to the rest of the cell; the position of the front flagellum marked by a row of dots (sectioned Flimmer). Electron micrograph M190.9, magnification $\times 10,000$.
- FIG. 16. L.S. of an expanded spermatozoid to show surface membranes and thin layer of cytoplasm outside the nucleus. Electron micrograph M183.27, magnification $\times 15,000$.

PLATE VI

- FIG. 17. T.S. of a cell in the region of the eyespot showing the nucleus (*n*), a mitochondrion (*m*), the cell membrane, the fibrils continuing from the base of the proboscis (*p*), the chromatophore and eyespot (*e*). Electron micrograph H85D, magnification $\times 20,000$.
- FIG. 18. An oblique T.S. through a less well preserved cell; letters as in Fig. 17; the arrow points to a band of dark material visible again in Figs. 19 and 23. Electron micrograph H80C, magnification $\times 20,000$.
- FIG. 19. Another cell similar to that of Fig. 18 but seen from the opposite side. Electron micrograph H81B, magnification $\times 20,000$.

PLATE VII

- FIG. 20. L.S. through the front end of an uncoiled spermatozoid showing the basal body of the front flagellum and its attachment to the nucleus. Electron micrograph H95B, magnification $\times 25,000$.
- FIG. 21. A similar cell showing the L.S. of the basal body of the hind flagellum. Electron micrograph H28A, magnification $\times 25,000$.
- FIG. 22. T.S. of a hind flagellum in the region of the eyespot to show the orientation of the fibrils and the band of suspending material (arrow); from another section of the cell of Fig. 18, Pl. VI, more highly magnified. Electron micrograph H80D, magnification $\times 30,000$.
- FIG. 23. T.S. of the tip of a cell to show the orientation of the fibrils at the base of the front flagellum. Electron micrograph H26D, magnification $\times 30,000$.
- FIG. 24. T.S. through the body of an expanded cell behind the eyespot showing the nucleus, two mitochondria with characteristic tubular 'cristae', the body membrane, the descending fibrils of the proboscis and traces of some other unidentified cell inclusions. Electron micrograph H83D, magnification $\times 30,000$.

PLATE VIII

- FIG. 25. T.S. of coiled cell in the region of the basal bodies (for place of section see diagram, Text-fig. 5). Electron micrograph H63E, magnification $\times 20,000$.
- FIG. 26. T.S. of a coiled cell in the region of the eyespot showing the parts as labelled in Fig. 27. Electron micrograph H30A, magnification $\times 20,000$.
- FIG. 27. Tangential section of a coiled spermatozoid showing the eyespot (*e*) and chromatophore, two mitochondria (*m*) but only the tip of the nucleus (*n*). Electron micrograph H64E, magnification $\times 20,000$.
- FIG. 28. Longitudinal section through a coiled spermatozoid. For further explanation see Text-Fig. 5 and Pl. IX, Fig. 31. Electron micrograph H64B, magnification $\times 20,000$.

PLATE IX

- FIG. 29. Part of a T.S. of a cell in the region of the eyespot to show the membranes covering the pigment compartments; *b.m.* body membrane, *c.m.* chromatophore membrane, *n* nucleus, *m* mitochondrion. Electron micrograph H62B, magnification $\times 40,000$.
- FIG. 30. Section parallel to cell surface passing transversely through the pigment compartments of the eyespot. Electron micrograph H60B, magnification $\times 30,000$.
- FIG. 31. More highly magnified part of Pl. VIII, Fig. 28, to show the relation of the hind flagellum to the eyespot. Electron micrograph H64B, magnification $\times 30,000$.

PLATE X

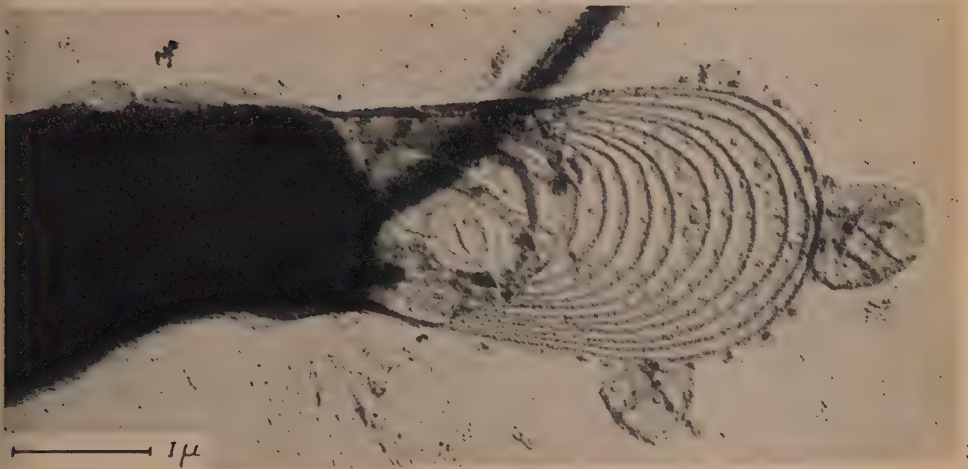
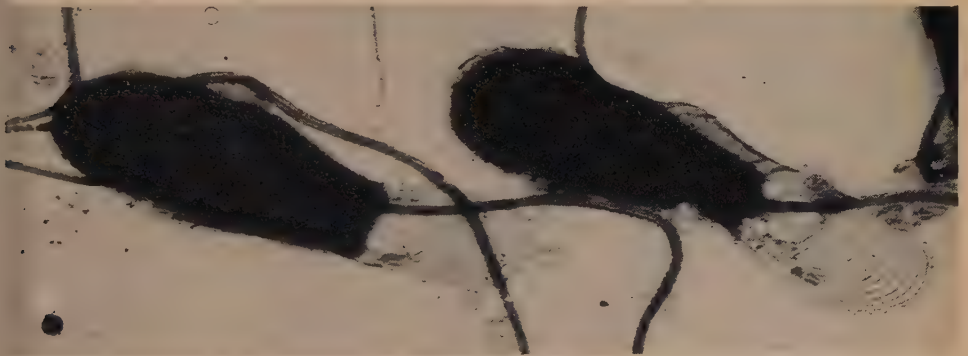
- FIGS. 32-35. Four different levels through the same cell selected from a longer series of transverse sections in the region of the ciliary bases. For further explanation see text, p. 426. Electron micrograph H91B, H90D, H89E, H89C, magnifications $\times 20,000$.
- FIG. 36. T.S. of a front flagellum showing the plane of symmetry (vertical to page) in relation to the origin of one of the lateral lines of hairs. Electron micrograph H83A, magnification $\times c. 40,000$.
- FIGS. 37-38. Two adjacent sections through a proboscis and front flagellum; the plane of symmetry of the flagellum transverse to the page. Electron micrographs H78B and H80A, magnification $\times c. 40,000$.
- FIG. 39. T.S. of a cell above the ciliary bases to show the fibres of the proboscis (*p*) on each side and the strands *s'* (see text, p. 426). Electron micrograph H83A, magnification $\times 30,000$.

PLATE XI

- FIGS. 40-41. Two sections at different levels through the tip of a cell to show the structure of the proboscis in L.S. (see text, p. 425). Electron micrographs H81C, H75D, magnification $\times 30,000$.
- FIGS. 42-43. Two transverse sections through one cell in the region of the ciliary bases, to show details of the origin of proboscis fibrils (see text, p. 426, and Text-fig. 2). Electron micrographs H79E and C, magnification $\times 30,000$.

PLATE XII

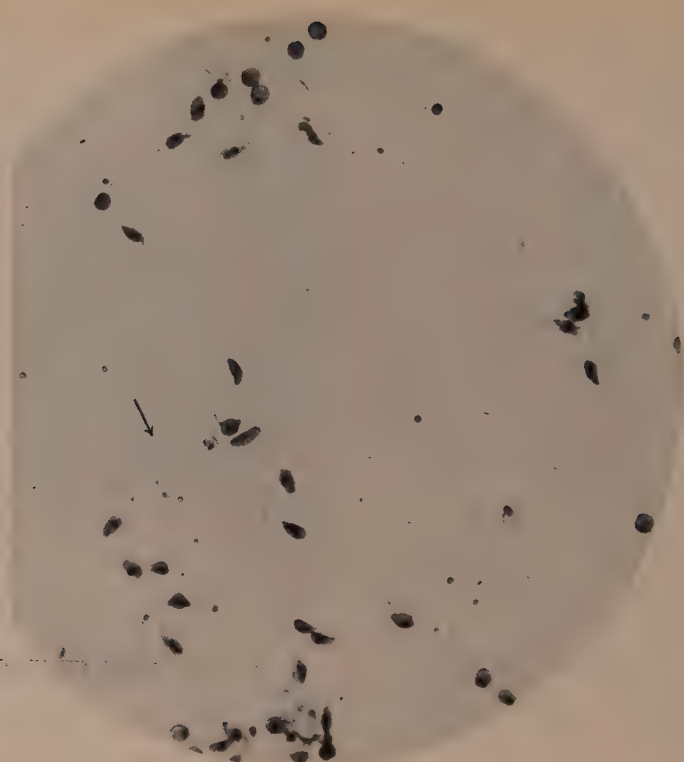
- FIG. 44. L.S. through ciliary bases to show the origin of the strand *s* between them. Electron micrograph H65E, magnification $\times 20,000$.
- FIG. 45. Whole mount of the ciliary apparatus from a dismembered cell (vapour fixation), *f.f.* front flagellum, *h.f.* hind flagellum, *p* proboscis, *s* strand to eyespot. Electron micrograph M86.13, magnification $\times 5,000$.
- FIG. 46. Whole mount of central region of ciliary apparatus from a dismembered cell (pH 8.2); letters as Fig. 45, for *s'* see text, p. 426. Electron micrograph, M145.9, reversed print, magnification $\times 15,000$.



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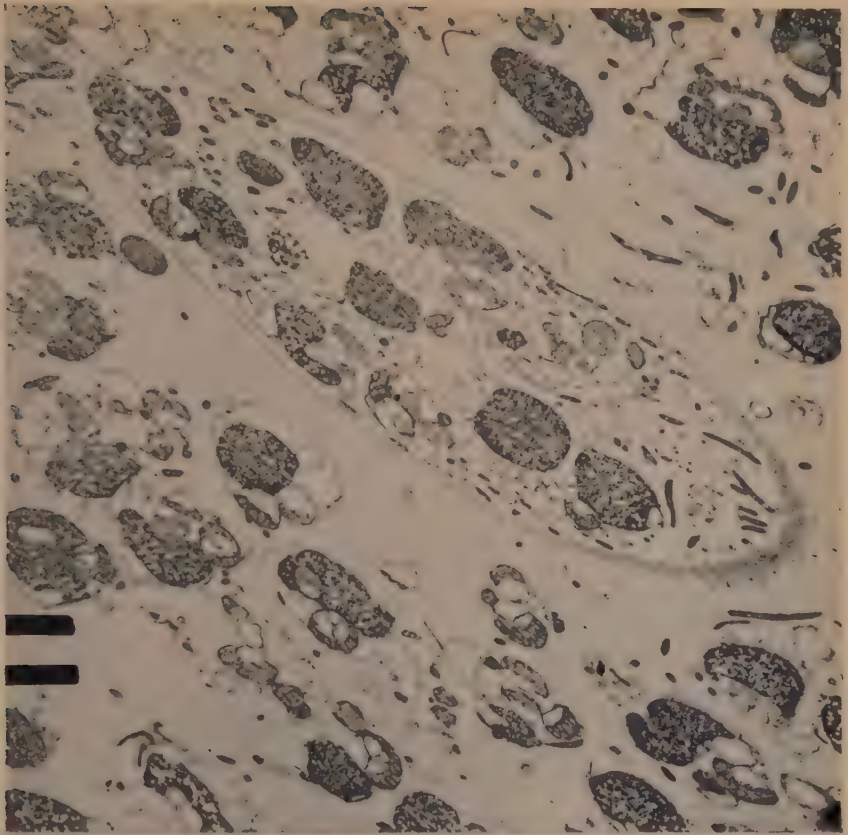
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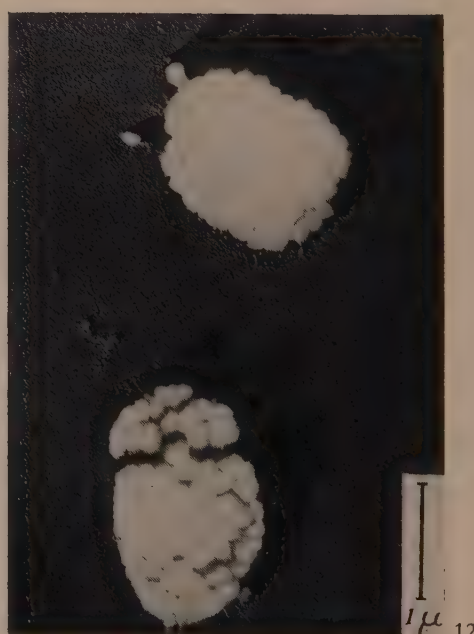
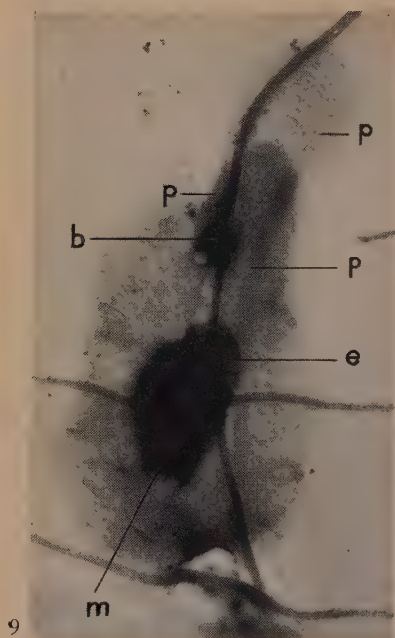
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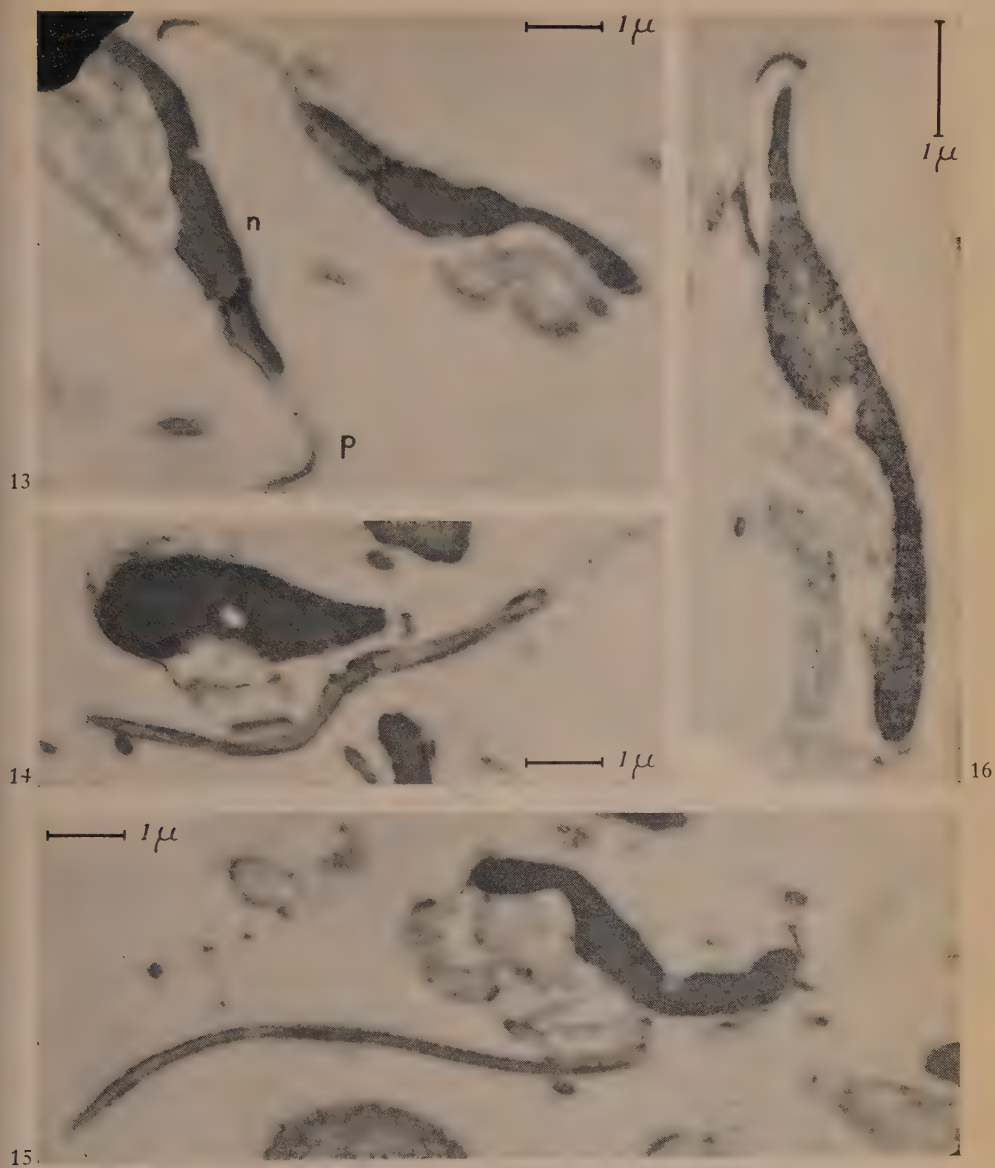
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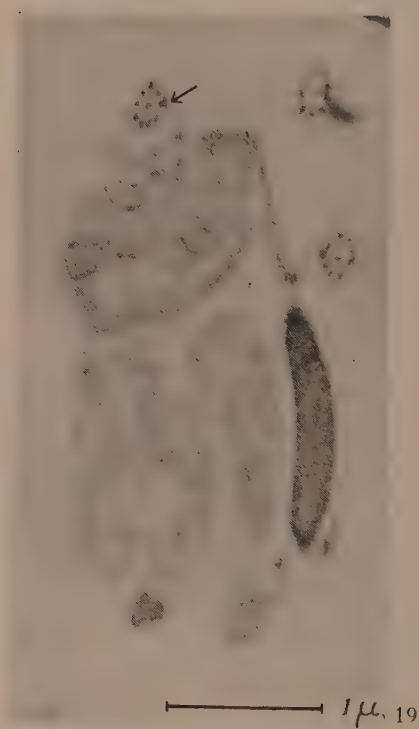
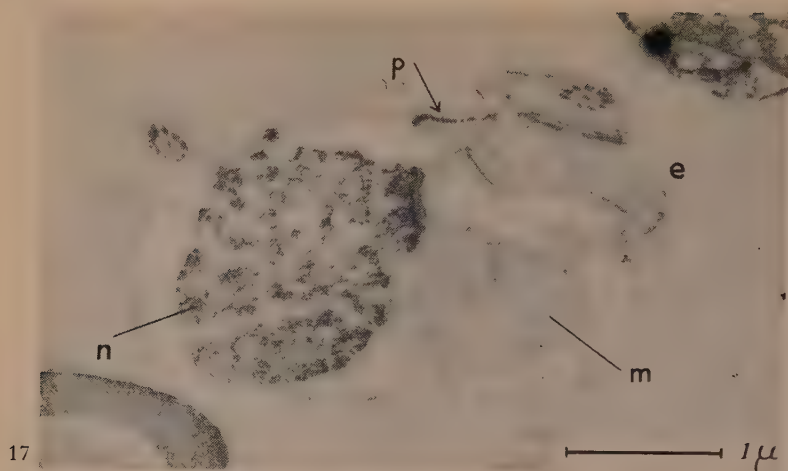
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I. MANTON and B. CLARKE—PLATE IV



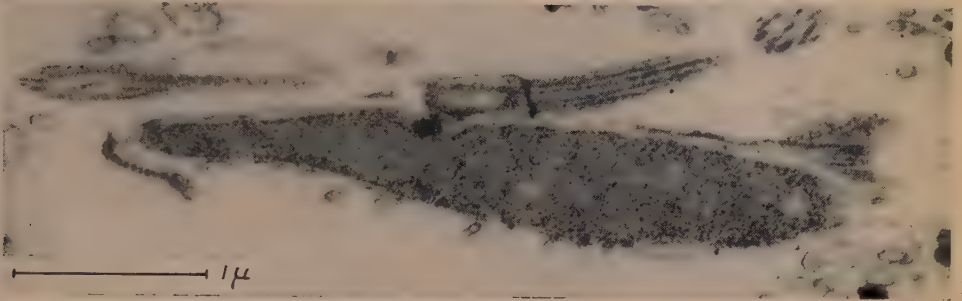
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I. MANTON and B. CLARKE—PLATE VI



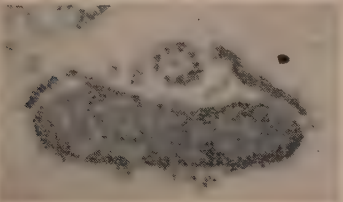
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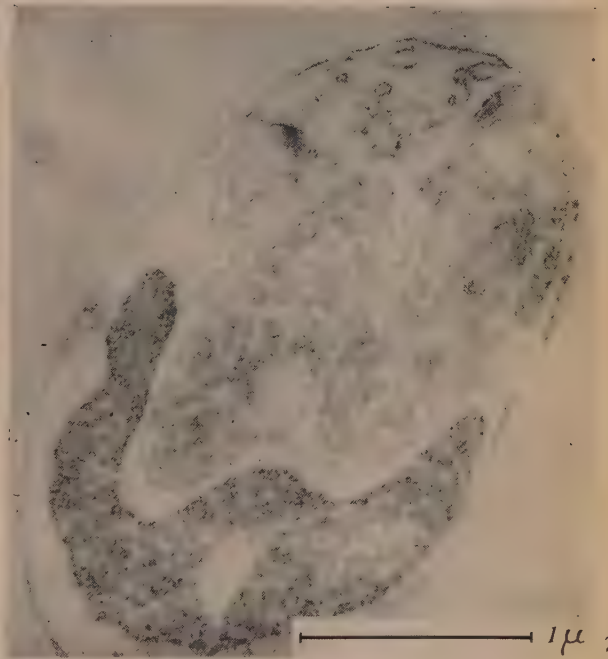
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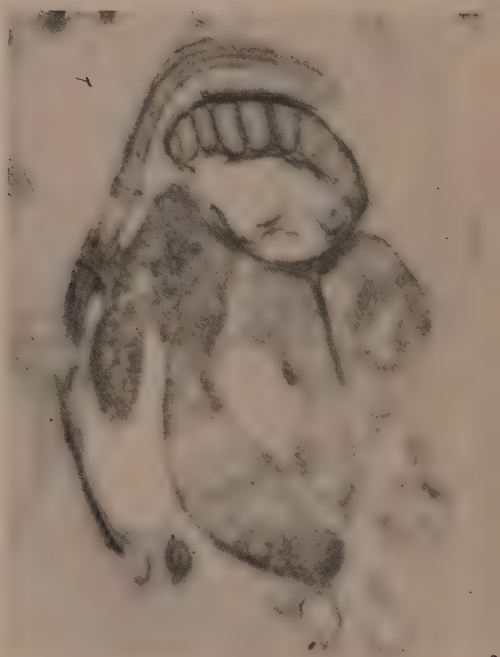
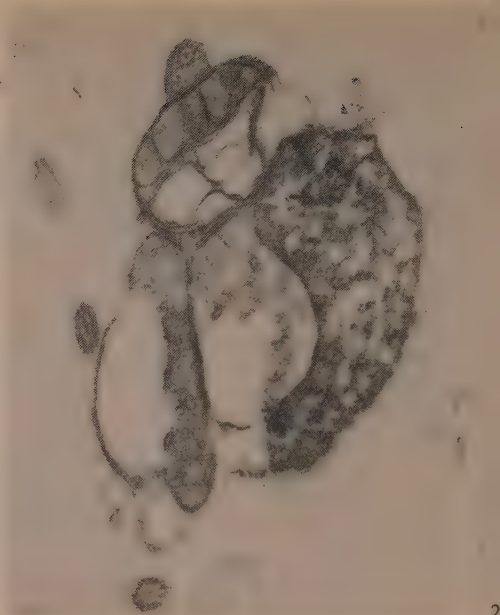
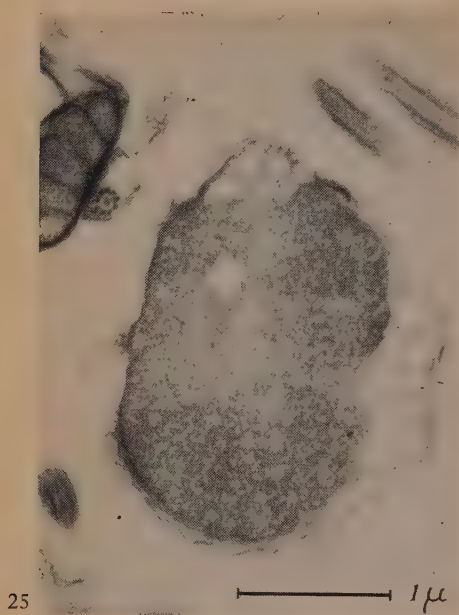
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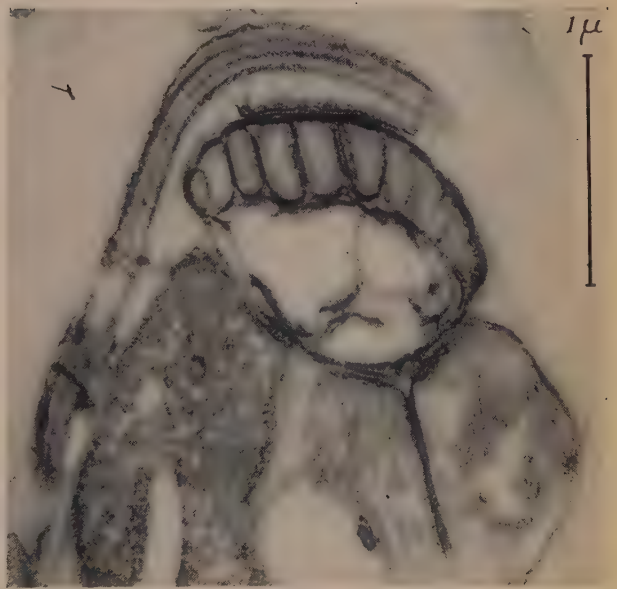
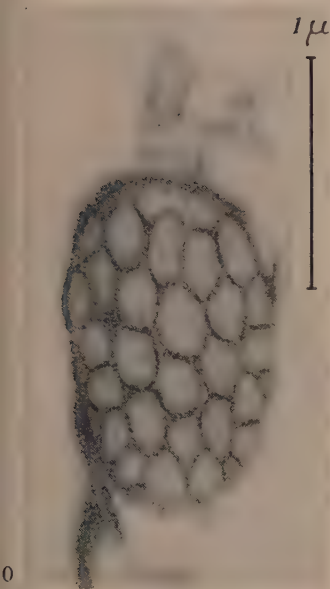
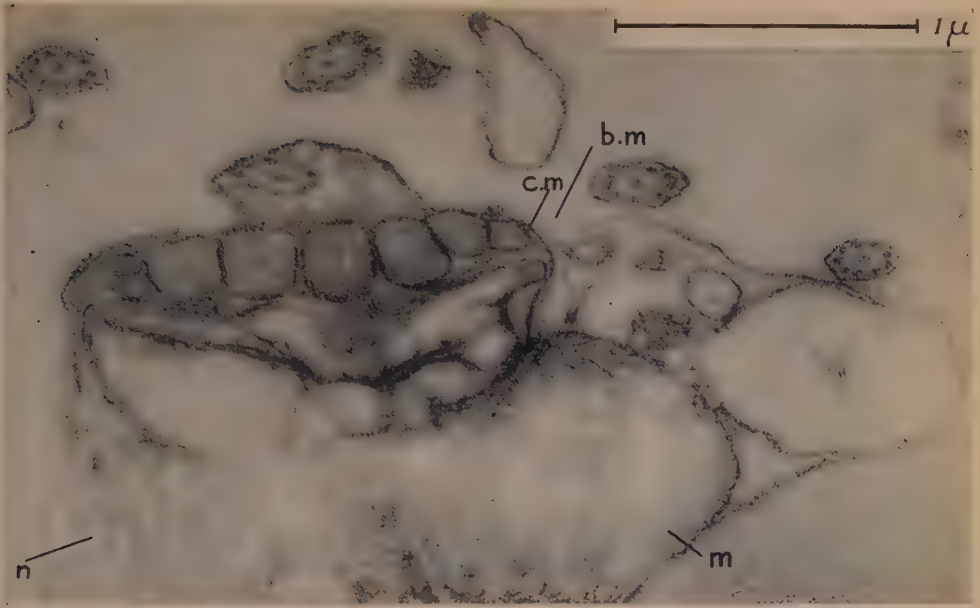


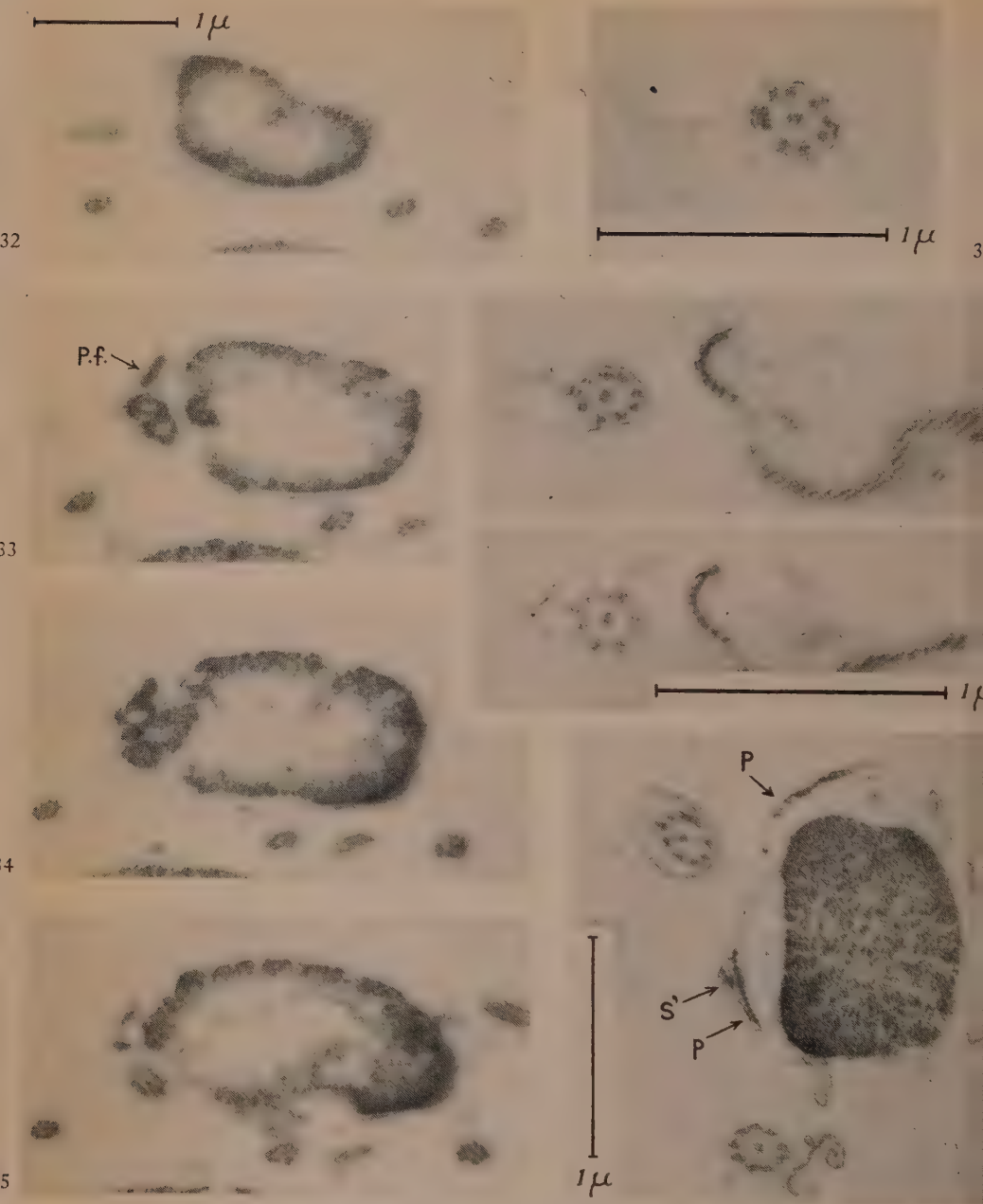
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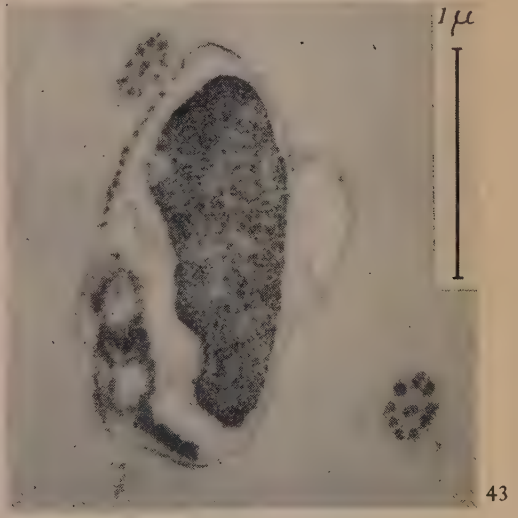
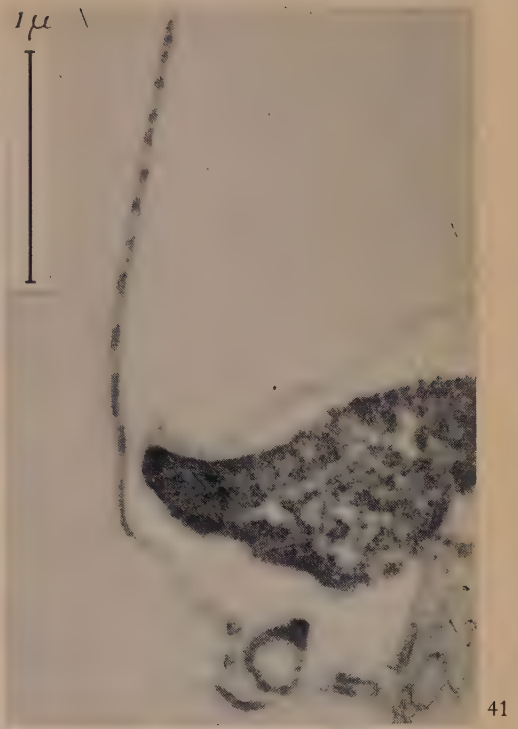
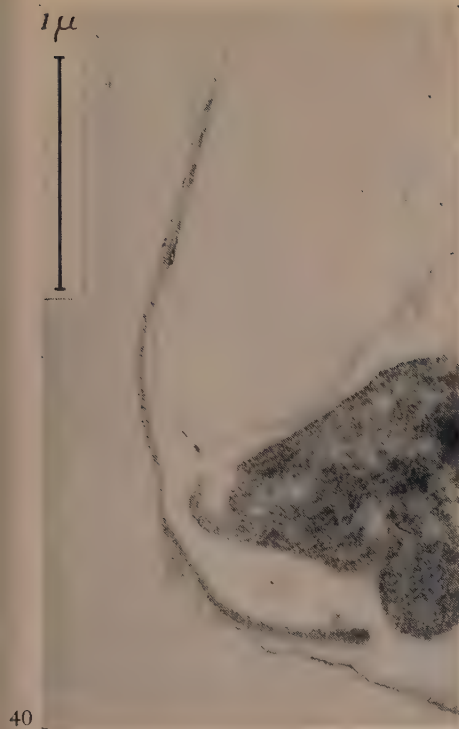
24



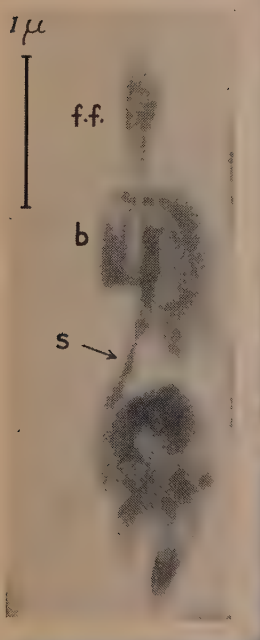
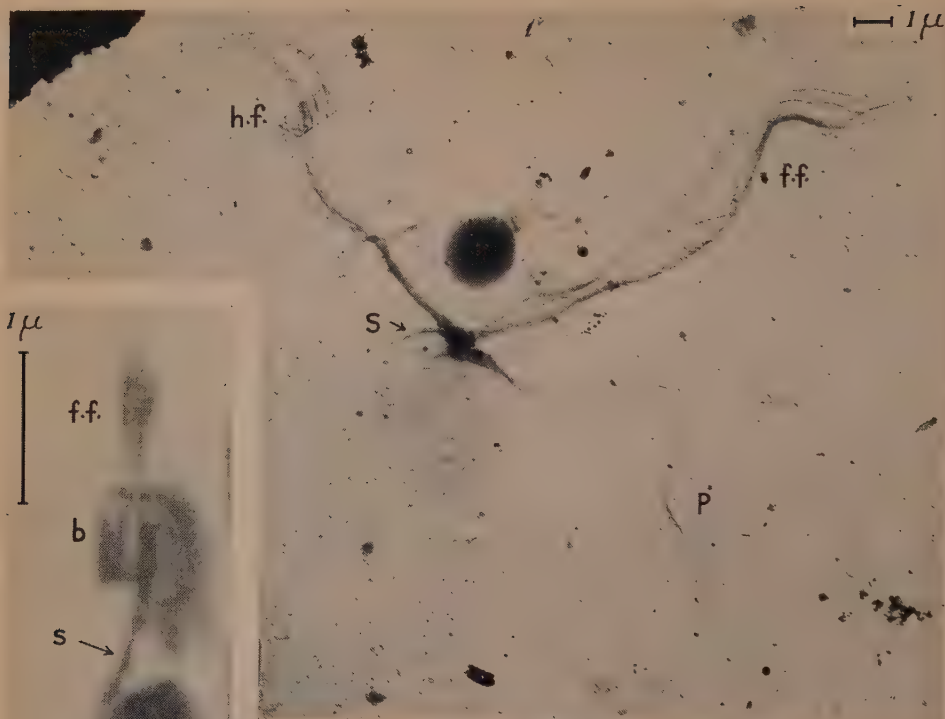




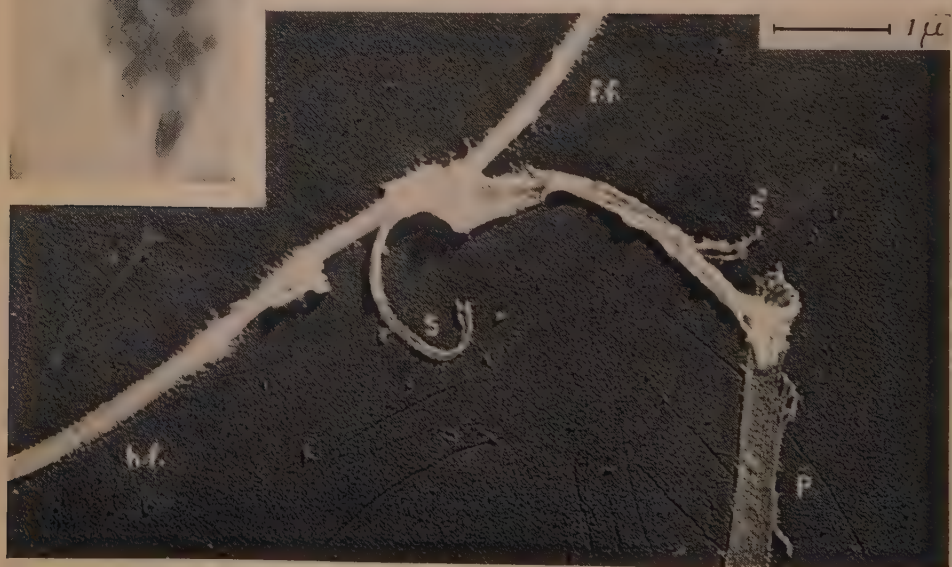
I. MANTON and B. CLARKE—PLATE X



I. MANTON and B. CLARKE—PLATE XI



44



45

46

